

The ins of the striatum: Utilizing chemogenetics to define the contribution of
cortical and thalamic afferents during addiction behaviors

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Abstract

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Addiction is a chronic neuropsychiatric disorder accompanied by high rates of recidivism that lacks effective treatment, part of which may be due to an incomplete understanding of the brain circuits mediating addiction. Cortico-basal ganglia circuitry is a complex, interconnected network regulating addiction. Aberrant glutamatergic signaling in NAc is particularly important for the development and persistence of addiction, and NAc neurons receive glutamatergic innervation from many structures, with prefrontal cortex (PFC) and midline and intralaminar thalamic nuclei (MTN) inputs predominating. Recently we developed a viral mediated gene transfer approach combined with chemogenetics that allows us to selectively activate $G_{i/o}$ -signaling cascades to reduce neuronal activity in specifically prefrontal cortex (PFC) or MTN NAc

afferents during addiction-related behaviors. Thus, the overall goal of this dissertation was to utilize these novel techniques to more clearly define the role of PFC or MTN neurons projecting to the NAc in the regulation of psychomotor sensitization, drug-self administration, and drug-seeking behaviors in rats.

We found that reducing neuronal activity of PFC neurons projecting to NAc attenuated the development of amphetamine sensitization. However, attenuating activity of these neurons during sensitization enhanced conditioned responses during a subsequent challenge session. Furthermore, our corticostriatal manipulation did not alter drug-taking during self-administration, but led to slower rates of extinction and enhanced responding following a priming injection of cocaine. We normalized responding following inhibition of corticostriatal afferents immediately prior to the drug-primed reinstatement test. These results demonstrate that corticostriatal afferents modulate responsiveness to psychostimulant drugs and drug-associated stimuli.

Considerably less is known about the relative contribution of MTN to relapse behaviors compared to other sources of NAc glutamate, despite sending dense projections to NAc. First, I demonstrate that reducing activity of MTN attenuates both cue-induced and drug-primed reinstatement of cocaine-seeking, which establishes a role of MTN in relapse behaviors. Then I show that dampening activity of specifically anterior MTN neurons projecting to NAc (MTN-NAc) abolished drug-prime reinstatement, but enhanced cue-induced reinstatement. We found no effect of the same manipulation in posterior MTN-NAc during either reinstatement behavior. These results demonstrate MTN mediate relapse behavior, and MTN-NAc may be particularly important for regulating responses to drug-associated stimuli.

Dedicated to:

My family for providing unwavering support and unconditional love for all of these years,

My loved ones who have struggled with addiction and conquered it,

and

Those still suffering from addiction, who feel there is no end to their affliction.

May this work provide hope that there are people on your side who have devoted their careers to understanding how drugs impact the brain. We are working towards a cure.

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Chapter 1

Introduction

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Yager LM, Garcia AF, Wunsch AM, Ferguson SM (2015). The ins and outs of the striatum: Role in drug addiction. *Neuroscience*, 301: 529-541.

The parts of the review included in this introduction were written by AMW. LMY, AFG, and SMF wrote other parts of this review article and provided manuscript edits.

1.1 Cocaine Addiction

Cocaine use has a long history. South Americans have chewed cocoa leaves, the natural source of cocaine, for its stimulant properties for thousands of years. Furthermore, cocaine was included in early medications for a variety of illness and used to treat pain in early surgical settings. In fact, use was so prevalent that it was once included as an ingredient in Coca Cola (Goldstein et al., 2004). However, today cocaine is classified as a schedule II drug because of its high potential for abuse and is considered a highly addictive stimulant. The National Survey on Drug Use and Health (2014) revealed that 1.5 million Americans are current cocaine users, and 913,000 Americans meet the Diagnostic and Statistical Manual (DSM) 4 criteria for a substance use disorder. Current DSM 5 criteria include tolerance, withdrawal, craving, significant time spent seeking and obtaining the drug, continued use despite negative consequences, and interference with daily activities (ie. work, school, or home responsibilities). Unfortunately, former cocaine users are at a high risk for relapse, even after long periods of abstinence. Triggers for relapse include exposure to cues associated with cocaine use, which can lead to strong cravings for the drug that ultimately lead to cocaine-seeking and cocaine intake (Hyman et al., 2006; Wolf, 2016).

Heavy cocaine use can cause profound changes in the brain. Cocaine binds to the dopamine transporter, which leads to an increase in the concentration of dopamine in the synapse. This increase in dopamine is thought to lead to the euphoric and rewarding effects of cocaine (Baik, 2013). Thus the corticomesolimbic dopamine system, which originates in the ventral tegmental area and extends to the nucleus accumbens, has been identified as the critical circuit associated with cocaine addiction.

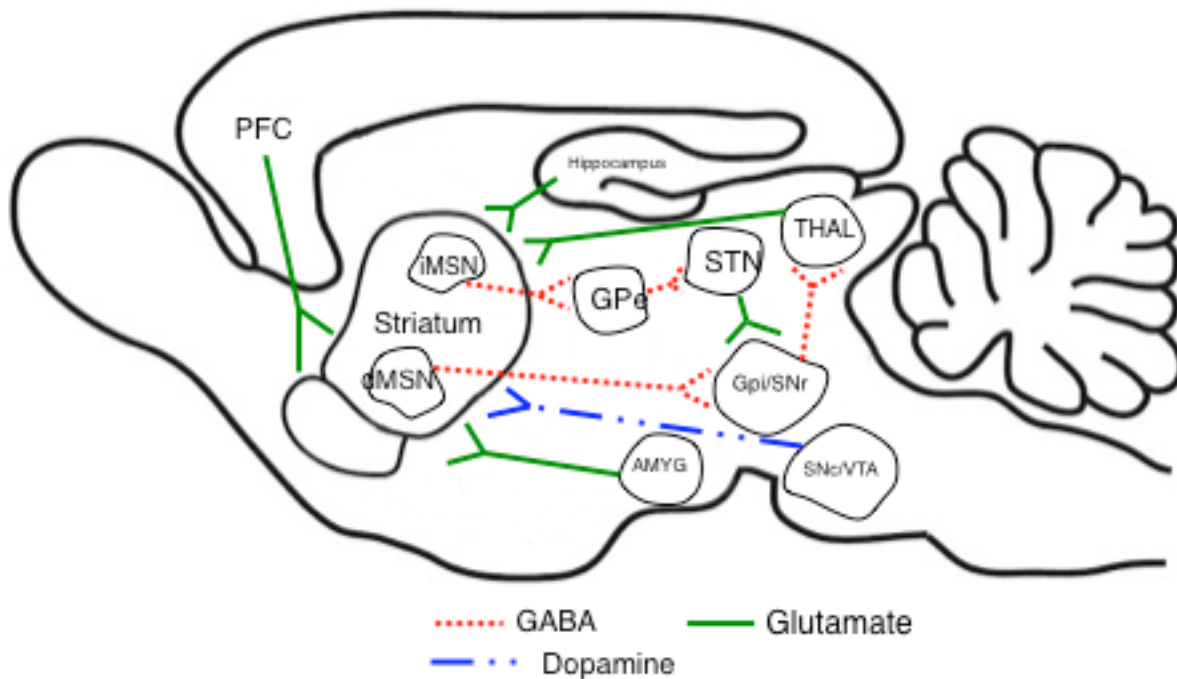


Figure 1.1. Cortico-basal ganglia circuit associated with addiction. Simplified view of the striatum and its afferents and efferents. The striatum receives glutamatergic inputs (denoted in green) from the prefrontal cortex (PFC), hippocampus, thalamus (THAL), and amygdala (AMYG). Dopaminergic inputs (dashed blue line) originate in the substantia nigra pars compacta/ventral tegmental area (SNc/VTA). Direct pathway medium spiny neurons (dMSNs) project directly to globus pallidus internal/substantia nigra pars reticulata (Gpi/SNr) whereas indirect pathway medium spiny neurons (iMSNs) project to Gpi/SNr via the subthalamic nucleus (STN). GABAergic inhibitory projections are indicated with a dotted red line.

Besides altering dopamine transmission in the nucleus accumbens, extended cocaine use also leads to profound changes in excitatory glutamatergic transmission. Thus therapeutic interventions that reduce cocaine-induced changes in plasticity in the nucleus accumbens may be effective in preventing relapse (Steketee & Kalivas, 2011; Wolf, 2016). However, nucleus accumbens glutamatergic afferents arise from several structures in the circuit, and it has been difficult to target manipulations to specific glutamatergic afferents that would allow us to define the role of specific afferents in addiction (Figure 1.1; for review Yager et al., 2015). Thus the dearth of treatment success may be due in part to an incomplete understanding of how this complex circuit

regulates addiction. The overall goal of this thesis will be to define the role of glutamatergic corticostriatal and thalamostriatal neurons in addiction-related behaviors. I will use recently developed chemogenetic and viral techniques to reduce activity of these neurons in rats during psychomotor sensitization, drug-taking, or reinstatement of drug-seeking paradigms.

This introduction will focus on recent studies that utilize input specific techniques to explore the role of specific sets of striatal glutamergic afferents in psychostimulant addiction-related behaviors. I will first discuss the rodent models of addiction relevant to this thesis project, psychomotor sensitization to amphetamine, cocaine self-administration, and reinstatement models of relapse. Then I summarize behavioral studies that demonstrated the importance of glutamatergic signaling in nucleus accumbens (NAc) during addiction-related behaviors. Because NAc receives glutamatergic inputs from several sources, I discuss recent studies demonstrating that specific glutamatergic inputs drive distinct aspects of addiction and associated cocaine-induced alterations in plasticity in NAc. Lastly I discuss the viral and chemogenetic techniques used in this thesis that allow for transient dampening of neuronal activity during addiction behaviors in specific glutamatergic afferents in rats.

1.2 Animal Models of Addiction

Psychomotor sensitization. Psychomotor sensitization is a commonly used rodent model to study the neural substrates of addiction. It is characterized by increases in locomotor activity and stereotyped behaviors (ie. repetitive movements such as head bobbing, grooming, or jumping occurring in a confined space) following repeated

injections of the same dose of a drug of abuse, such as amphetamine, and by enhanced behavioral responses to drug re-exposure following withdrawal (Robinson & Becker, 1986; Segal & Mandell, 1974). Several lines of evidence support this idea of sensitization as a model of addiction-related behaviors. Psychotomimetic symptoms, eye blink rates, and energy ratings sensitize in humans, rodents exhibit sensitized behaviors for up to one year following the last drug exposure, and rats that have sensitized show facilitated acquisition of self-administration (Ferrario & Robinson, 2007; Leyton, 2007; Paulson et al., 1991). Most importantly, sensitization is accompanied by morphological and neurochemical alterations such as increases in dendritic spine density and enduring changes in dopamine and glutamate transmission within the C-BG circuit that also occur following self-administration and are thought to underlie the development and maintenance of addiction in humans (Ferrario et al., 2005; Robinson & Kolb, 1999; Steketee & Kalivas, 2011). Thus, sensitization provides a simple, efficient method for determining the mechanisms that underlie the addiction.

Cocaine self-administration and reinstatement. Short access cocaine self-administration is another commonly used pre-clinical rodent model of addiction. During self-administration, rats learn that an action (i.e. lever press or nosepoke) leads to the delivery of an intravenous injection of a drug, such as cocaine. Rats rapidly learn to show a preference for the active lever (which is paired with cocaine delivery), over the inactive lever (on which presses do not have any programmed consequences). Typically rats have continuous access to cocaine for the duration of the behavior session (ranging from 1-3 hours), and the only limit imposed on the number of infusions that can be taken is a time-out period following drug delivery (commonly 20 seconds).

Rats undergo at least 10 sessions of self-administration to ensure robust drug-taking behavior (Belin-Rauscent et al., 2016; Everitt, 2014; Everitt & Robbins, 2005; Panlilio & Goldberg, 2007). One primary advantage of this instrumental learning paradigm is that rats voluntarily self-administer cocaine. Furthermore, cocaine delivery can be paired with unconditioned stimuli (ie. cue light or tone) that, through Pavlovian learning processes, can lead to enhanced incentive value applied to these cues and lead to reinvigoration of drug-seeking (ie. increase in active lever presses) when re-exposed to these cues following extinction training or abstinence. This reinstatement of drug-seeking occurs not only to conditioned cues, but also to exposure to low doses of the drug itself, and stressors, all of which are thought to contribute to relapse in human addicts (Epstein et al., 2006; Marchant et al., 2013; Wolf, 2016). Thus, short access cocaine self-administration has been a critical tool used to trace the role of cortico-basal ganglia circuitry in drug-taking and drug-seeking behaviors.

More recently it has been argued that short access to cocaine self-administration may not fully capture the compulsive nature of addiction. Newer models have been developed to capture key aspects of addiction as outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychological Association, 2000). Criteria include an enhanced motivation for drug-taking, escalation of drug intake, and resistance to extinction and punishment, and increased vulnerability to reinstatement of drug-seeking (Ahmed, 2012; Everitt, 2014; Lesscher & Vanderschuren, 2012). In addition, unlike short access paradigms, humans will binge on cocaine, having long periods of time in between sessions of cocaine intake (Dackis & O'Brien, 2001).

Thus, short access does not accurately reflect the pattern of drug-intake in human addicts.

Recently, an intermittent access to self-administration (IntA) paradigm was developed to more closely model the bingeing pattern of drug intake (Calipari et al., 2014; Zimmer et al., 2012b). Rats have 5 minutes of unlimited access to cocaine followed by a 25-minute time-out period. This pattern of self-administration leads to a “spikes” in the concentration of cocaine in the brain, which may more closely reflect binge-like drug-taking behavior of human addicts (Martin-Garcia et al., 2014; Zimmer et al., 2012b). Importantly IntA leads to an enhanced motivation to take cocaine compared to rats trained on a short access self-administration paradigm, suggesting it may predispose rats towards exhibiting critical markers of addictive behaviors (Calipari et al., 2014; Zimmer et al., 2012b). Additionally, rats that exhibit a higher frequency to self-administer (ie. short inter-infusion interval) during a modified IntA paradigm have higher rates of cocaine-primed reinstatement of drug-seeking, further supporting IntA as modeling traits associated with addiction (Martin-Garcia et al., 2014). Studies are still warranted as to whether IntA leads to escalation of cocaine intake, enhanced extinction, or heightened cue-induced or stress-primed reinstatement.

1.3 Alterations in glutamatergic signaling in nucleus accumbens underlie addiction

A majority of the prior research on addiction circuitry has used lesion, pharmacological, and microdialysis methods to demonstrate the importance of glutamatergic neurotransmission within the striatum in addiction-related behaviors. The nucleus accumbens (NAc; or ventral striatum) regulates several aspects of addiction,

Addiction Model	Description	Citation
Incubation of craving	Increase in drug craving dependent on the length of abstinence. Rodents learn to self-administer drug and then undergo a period of drug withdrawal. Subsequent exposure to drug-related cues reinstates operant responding. More drug seeking is observed with longer periods of abstinence	Pickens (2011) Marchant (2013) Li (2015)
Progressive Ratio	Used to assess the motivation to take drugs. Progressive ratio testing consists of increasing the response requirement for each subsequent infusion. The point at which an animal ceases to respond because the response requirement requires more effort than the reward is worth is referred to as the breakpoint. Higher breakpoints indicate higher levels of motivation to obtain drug.	Stafford (1998)
Psychomotor Sensitization	Form of drug-induced plasticity that can measure long-term effects of drug exposure. Rodents receive repeated non-contingent injections of drug over several days, and responses (i.e., locomotor activity or stereotypies) to drugs increases. Following a period of abstinence, animals receive another drug injection and enhanced response persists.	Steketee (2011) Robinson (2008) Robinson (2000)
Reinstatement of drug-seeking	Model of relapse. Rodents learn to self-administer drug which is followed by extinction of responding. Subsequent presentation of drug-paired stimuli (e.g., cue, drug, or context) or a stressor restores operant responding.	Marchant (2013) Bossert (2013) Epstein (2006)
Self Administration	Response-dependent administration of drug. Rodents learn that performing an operant response (e.g., lever press or nosepoke) on the active lever/port results in drug infusion whereas responses on inactive lever/port does not. Responses on active lever/port are higher than on inactive lever/port in animals that learn to self-administer drug.	Belin-Rauscent (2015) Panlilo (2007) Everitt (2005)

Table 1.1. Glossary of addiction-related behavioral terminology used.

Citations are listed for additional information.

including expression of psychomotor sensitization, incubation of drug-craving, and reinstatement behaviors (Everitt et al., 1999; Sesack & Grace, 2009; Steketee &

Kalivas, 2011; Wolf, 2016). Increases in the concentration of glutamate and immediate early gene expression (a marker of neuronal activity) within the NAc have been demonstrated following acute and chronic drug exposure and behaviors associated with relapse (Harlan & Garcia, 1998; Torregrossa & Kalivas, 2008). Additionally, similar increases concentration of glutamate and neuronal activation have been observed in glutamatergic neurons projecting to the striatum, such as the prefrontal cortex (PFC), midline and intralaminar nuclei (MTN), amygdala (AMYG), and hippocampus (HIPPO) (Ciccocioppo et al., 2001; Kelley et al., 2005; Kufahl et al., 2009; Neisewander et al., 2000; Ostrander et al., 2003). Lesions and pharmacological manipulations have confirmed a causal role for all these regions in psychomotor sensitization and drug-taking and -seeking behaviors (Capriles et al., 2002; Y. Li et al., 1999; McFarland & Kalivas, 2001; McFarland et al., 2003; See et al., 2007; Tzschentke & Schmidt, 1999; 2000; Weissenborn et al., 1998; Wolf et al., 1995). Importantly, glutamatergic projections into the striatum also appear to be critical for reinstatement of drug-seeking as pharmacological blockade of glutamate in the NAc prevents relapse (Ikemoto & Bonci, 2014; Kalivas et al., 2008; Park et al., 2002; Pierce & Wolf, 2013; Wolf, 2010).

However, there is ambiguity regarding the exact nature of the contribution of each of these regions to addiction. This is in part due to the non-specific nature of the techniques that were utilized to examine the role of these regions. Thus, while lesions and pharmacological manipulations have provided insight into the broad nature of cortico-basal ganglia circuitry regulation of addiction, these techniques lack both temporal and cellular specificity. For example, these approaches cannot parse apart the function of specific afferent projections. Given the vast interconnectivity of this circuit,

isolating the role of individual projections is critical for gaining a complete understanding of the neural mechanisms underlying addiction. Fortunately, the emergence of recent molecular, viral, and chemogenetic and optogenetic techniques have provided new methods for more precisely exploring the relationship between neural activity and addiction-related behaviors. In the following sections I will discuss recent studies that utilize these techniques to parse apart the role of striatal afferents in regulating addiction-like behaviors.

1.4 Role of striatal glutamatergic afferents in addiction

The striatum not only receives dense dopamine projections from the ventral tegmental area and substantia nigra pars reticulata, but it also receives innervation from glutamatergic projection neurons originating in multiple brain regions (Figure 1.1). Each of these glutamatergic inputs is thought to regulate distinct aspects of the behaviors associated with addiction (Sesack & Grace, 2009). The cortex is the predominant source of striatal glutamate and corticostriatal projections display topographic specificity, such that more dorsolateral regions of the striatum receive projections from sensorimotor cortex and more ventromedial portions of the striatum receive projections from associative cortex, including PFC (Groenewegen et al., 1990; Koob & Volkow, 2009; McGeorge & Faull, 1989; Sesack & Grace, 2009; Wall et al., 2013). Within the NAc, the PFC projections can be further subdivided with the infralimbic region projecting primarily to the NAc shell and the prelimbic region projecting primarily to the NAc core (Brog et al., 1993; Sesack et al., 1989).

The second largest source of striatal glutamate originates in the thalamus (Lei et al., 2013; Wall et al., 2013). MTN including the paraventricular nucleus (PVT), mediodorsal (MD), central median (CM), intermediodorsal (IMD), and parafasicular nuclei send dense glutamatergic projections to the striatum and synapse directly onto striatal medium spiny neurons (Berendse & Groenewegen, 1990; Haber & Calzavara, 2009; Lei et al., 2013; Li & Kirouac, 2007; Wall et al., 2013). Finally, the AMYG and HIPPO provide glutamatergic inputs mostly to NAc and both of these projections appear to synapse primarily on direct pathway medium spiny neurons (Britt et al., 2012; Groenewegen & Trimble, 2007; MacAskill et al., 2014; MacAskill et al., 2012; Pascoli et al., 2011; Wall et al., 2013). Although new technologies are being used to refine the role of each of these sources of striatal glutamate in addiction-related behaviors, in this section we will focus on the studies that specifically probed the role of the glutamatergic afferents into the striatum.

Prefrontal Cortex. Dysregulation of the PFC following drug use is widely believed to underlie the loss of inhibitory control seen in drug addicts and is a primary contributing factor in relapse (Goldstein & Volkow, 2011; Kalivas, 2009). Work in rodent models has found that the prelimbic and infralimbic areas of the PFC are particularly important for regulating drug-seeking and the expression of addiction-related behaviors such as psychomotor sensitization and discrimination between levers during self-administration paradigms (Francis et al., 2014; Moorman, James et al., 2014; Pascoli et al., 2014; Seamans et al., 2008).

Although the PFC has widespread and reciprocal projections within the cortico-basal ganglia circuit, recent studies support the idea that both drug-induced

neuroplasticity in MSNs and drug-induced behaviors are modulated specifically by PFC afferents to the NAc shell. For example, infralimbic inputs to the NAc shell were found to undergo silent synapse based remodeling following withdrawal from cocaine self-administration that was dependent on the insertion of calcium-permeable AMPA receptors, and reversal of this silent-synapse based remodeling via optical stimulation enhanced cue-induced cocaine seeking (i.e., incubation of craving) (Ma et al., 2014). Similarly, using optical stimulation of infralimbic terminals within the NAc shell of fluorescently-labeled dopamine D1 or dopamine D2 BAC transgenic mice, it was found that withdrawal from cocaine self-administration resulted in enhanced glutamatergic signaling in D1 MSNs (direct pathway medium spiny neurons, dMSN) but not D2 MSNs (indirect pathway medium spiny neurons, iMSN) that was also due to insertion of calcium-permeable AMPA receptors (Pascoli et al., 2014). Although using an optical stimulation LTD protocol to restore normal transmission selectively in these inputs prior to cue-induced reinstatement also increased lever responding, it did so non-discriminatively (Pascoli et al., 2014). Nonetheless, using this method to normalize transmission prior to a cocaine challenge was sufficient to block the expression and persistence of cocaine-induced locomotor sensitization (Pascoli et al., 2011).

Studies have also begun to explore the role of PFC inputs into the NAc core in addiction-related plasticity and behavior. Similar to the NAc shell, withdrawal from cocaine self-administration also produced silent synapse remodeling in prelimbic inputs to NAc core MSNs, but this occurred via a mechanism that was dependent on non-calcium permeable AMPA receptor insertion (Ma et al., 2014). Furthermore, reversal of this silent-synapse based remodeling via optical stimulation inhibited cue-induced

cocaine seeking (Ma et al., 2014). Likewise, optical inhibition of prelimbic afferents to the NAc core during reinstatement reduced cocaine seeking during both cocaine-primed reinstatement and cocaine-plus-cue-induced reinstatement (Stefanik et al., 2015; Stefanik et al., 2013).

It is likely that these post-synaptic neuroadaptations at corticostriatal synapses are at least partly due to drug-induced presynaptic alterations in neurotransmitter release. In support of this, optogenetic stimulation of PFC inputs to the NAc following either contingent or non-contingent cocaine administration revealed an increase in release probability at both short and long withdrawal periods, although the probability of neurotransmitter release was higher in mice that had self-administered cocaine compared to those that had received experimenter-administered drug (Suska et al., 2013).

Together, these studies support the idea of dissociable roles of PFC projections to the NAc core and shell in regulating addiction behavior and associated plasticity, although it is clear that the manner in which they do so is complex. In addition, it should be noted that changes in plasticity at corticostriatal synapses within the NAc are not always observed following repeated drug treatment (Britt et al., 2012). Multiple factors, including amount of drug intake, contingency of drug administration and withdrawal time influence the neurobiological changes that occur following drug use. Likewise, experimental variables such as optical stimulation parameters and the subset of cells that are targeted within a striatal or cortical region can also have a big impact on experimental outcomes. Thus, additional work to normalize such variables will be

required to gain a full understanding of the role cortical inputs into striatum play in addiction processes.

Thalamus. Although the thalamus densely innervates the striatum, it has largely been overlooked when studying the circuitry underlying addiction. Nonetheless, mounting evidence suggests that thalamic impairments contribute to the sensory processing and attentional deficits seen in addicts and may also modulate drug-craving and other addiction-related behaviors (James & Dayas, 2013; Koob & Volkow, 2009; Martin-Fardon & Boutrel, 2012). For example, cFos is upregulated in the PVT following an acute injection of either cocaine or amphetamine as well as after exposure to drug-associated cues or contexts (Deutch et al., 1998; Hamlin et al., 2009; James et al., 2011; Rotllant et al., 2010). Furthermore, lesions of the PVT enhance the acute locomotor response to an injection of cocaine, but block psychomotor sensitization and prevent context-induced reinstatement (Hamlin et al., 2009; Young & Deutch, 1998). MD has also been implicated in regulating responses to psychostimulants, as lesions of the MD attenuate cocaine intake during self-administration (Weissenborn et al., 1998). However, despite evidence indicating that MD neurons are active during cue-induced reinstatement, lesions of MD did not alter cocaine-primed or stress-induced reinstatement (James et al., 2011; McFarland & Kalivas, 2001). These data suggest that the various MITN may differentially regulate aspects of addiction-related behavior. However, even though the thalamus sends a strong glutamatergic projection to the striatum, there have been no studies to date utilizing more recent technology that allow for precise temporal and spatial control to define the specific role of these thalamic projections in addiction.

Amygdala. The AMYG is thought to regulate conditioned responses to cues associated with drug-taking (Bossert et al., 2013; Goldstein & Volkow, 2011; Kalivas et al., 2005). However, relatively few studies have directly assessed neurobiological changes in AMYG projections to striatum and their contribution to addiction-related behavior. Following a cocaine sensitization protocol, it was found that optogenetic stimulation of basolateral amygdala (BLA) inputs to the NAc shell selectively enhanced excitatory post-synaptic currents and increased spine density in direct pathway medium spiny neurons, suggesting that cocaine-induced alterations in direct pathway medium spiny neurons function and structure are due to an increase in the strength of BLA inputs to these neurons (MacAskill et al., 2014). However, other studies using optical stimulation of BLA inputs to the NAc shell following either cocaine self-administration or behavioral sensitization did not observe alterations in plasticity of striatal neurons (Pascoli et al., 2014; Suska et al., 2013). Nonetheless, these striatal glutamatergic afferents from the BLA appear to regulate incubation of craving and drug-seeking. For example, following cocaine self-administration and extinction, optical inhibition of BLA inputs to the NAc core during cue-induced reinstatement decreased cocaine-seeking (Stefanik, 2014). In addition, it was found that the incubation of cue-induced cocaine seeking normally seen following prolonged withdrawal could be blocked by an optical stimulation LTD protocol that reversed the maturation of silent synapses in BLA inputs to the NAc shell (Lee et al., 2013). Thus, while the extent to which BLA projections to the striatum contribute to changes in medium spiny neuron plasticity is unclear, there is ample evidence to support the role of these projections in regulation of the development of conditioned responses to cues associated with drug-taking as well as drug-craving.

Therefore, these projections are a strong candidate for therapeutic interventions that could mitigate relapse.

Hippocampus. The HIPP is thought to be involved in the formation of associations related to the context associated with drug-taking and may regulate the strength of responding during reinstatement (Francis et al., 2014; Koob & Volkow, 2009; Pascoli et al., 2014). Optical stimulation of ventral HIPP afferents to the NAc shell paired with electrophysiology recordings from fluorescently-labeled dopamine D1 receptor or dopamine D2 receptor BAC transgenic mice demonstrated that cocaine self-administration increased synaptic plasticity of these inputs onto dMSNs but not iMSNs (Pascoli et al., 2014). Alterations in synaptic plasticity within the NAc shell medium spiny neurons at ventral HIPPO synapses have also been observed following behavioral sensitization to cocaine (Britt et al., 2012). In addition, optical inhibition of ventral HIPPO terminals in the NAc shell blocked cocaine sensitization whereas optical stimulation enhanced this behavior (Britt et al., 2012). Furthermore, optical stimulation of these projections is rewarding in and of itself, as evidenced by the development of a CPP for a chamber paired with light stimulation (Britt et al., 2012). Finally, using an optical stimulation long term depression protocol to restore normal transmission selectively in HIPP inputs to the NAc shell prior to cue-induced reinstatement resulted in decreased cocaine-seeking (Pascoli et al., 2014). Additionally, cocaine-seeking was completely abolished when normal transmission was restored to both PFC and HIPPO inputs (Pascoli et al., 2014). Together, these studies provided evidence that drug exposure increases synaptic plasticity in ventral hippocampus projections to direct medium spiny neurons, which leads to an enhancement in contextual-mediated

associations within the drug-taking environment. However, in contrast to these findings, ventral HIPPO inputs to the NAc shell were actually dampened in direct medium spiny neurons three days after a sensitizing regimen of cocaine (MacAskill et al., 2014). These discrepant findings may reflect a differential involvement of ventral HIPPO in early versus late withdrawal, as well as differences in experimental parameters.

1.5 Using DREADDs to examine the role of cortical and thalamic striatal afferents in addiction-related behaviors

The studies described in this thesis will parse out the role of corticostriatal and thalamostriatal projection neurons in addiction-related behaviors using tools that allow for transient and reversible alteration of intracellular $G_{i/o}$ -signaling cascades. Specifically, viral-mediated gene transfer techniques were used to express $G_{i/o}$ -coupled DREADDs (hM4Di), in defined neuronal populations. Following expression of hM4Di in targeted neurons, activation occurred by systemic administration of the otherwise inert ligand clozapine-N-oxide (CNO). CNO has no affinity for endogenous receptors but is a potent agonist at hM4Di, which couples to $G_{i/o}$ to decrease cAMP activity and open GIRK potassium channels, thereby hyperpolarizing neurons and transiently inhibiting hM4Di -expressing neurons (Armbruster et al., 2007; Ferguson et al., 2010; Zhu & Roth, 2014). Activation of $G_{i/o}$ -signaling cascades via hM4Di in striatum or cortex also decreases local expression of immediate early genes such as cFos (Ferguson et al., 2010; Kerstetter et al., 2015). We chose to use tools to reduce activity of neurons for these experiments because studies that utilize stimulation may artificially induce activity

in pathways that do not normally participate in drug responses, thus leading to false identification of the involved neurons or regions.

To achieve expression of hM4Di in glutamatergic neurons that project to NAc, I used a cre-recombinase (cre) based flip-excision system (FLEX) (Atasoy et al., 2008; Schnütgen et al., 2003). An adeno-associated virus (AAV) expressing an inverted form of hM4Di fused to mCherry flanked by two, inverted loxP sites (FLEX- hM4Di) was injected into PFC or midline and intralaminar thalamic nuclei (MTN) of rats, and canine adenovirus (cAV) expressing cre (cAV-cre) was injected into NAc. cAV-cre is retrogradely transported to the cell bodies of any brain region that projects to the site of cAV infusion (Soudais et al., 2004). In the presence of cre, the loxP sites in FLEX vectors are excised, allowing for hM4Di expression under control of the synapsin I promoter. Consequently, we achieved stable expression of hM4Di specifically in PFC or MTN neurons projecting to NAc with this method. Our lab demonstrated robust transgene expression in PFC following injection of FLEX vectors into PFC and cAV-cre into NAc (Kerstetter et al., 2015). In addition, our lab found that increasing $G_{i/o}$ -signaling in cortical neurons projecting to NAc reduces cortical and striatal cFos induced by cocaine (Kerstetter et al., 2015). Thus, these data, together with previous studies, suggest that hM4Di activation reduces activity of PFC or MTN neurons and their downstream targets in NAc (Kerstetter et al., 2015; Zhu et al., 2016). By utilizing viral-mediated gene transfer techniques to express inhibitory, $G_{i/o}$ -coupled DREADDs in specific neuronal populations, I will be able to transiently reduce neuronal activity to assess the role of cortico- and thalamo-striatal neurons during addiction-related behaviors.

1.6 Thesis overview

The overall goal of the work described in this thesis is to utilize chemogenetic and viral mediated gene transfer techniques to explore how cortical and thalamic NAc afferents contribute to addiction-related behaviors. Specifically I will express hM4Di DREADD to reduce activity of corticostriatal, MTN, or thalamostriatal neurons during psychomotor sensitization, drug-taking, or reinstatement of drug-seeking. Chapter 2 describes work demonstrating that corticostriatal afferents modulate responsiveness to psychostimulants (amphetamine and cocaine) and psychostimulant-associated stimuli. Chapter 3 details a set of experiments that reveal a novel contribution of midline thalamic nuclei, in particular thalamostriatal neurons, to cocaine relapse. Finally I discuss the implications of this work in chapter 4.

Chapter 2

Corticostriatal afferents modulate responsiveness to psychostimulant drugs and drug-associated stimuli

*This chapter was published with minor reformatting as an article with the same title in *Neuropsychopharmacology*. Co-authors of this work included Kerry A. Kerstetter (co-first author), Kanichi G. Nakata, Elizabeth Donckels, John F. Neumaier, and Susan M. Ferguson. The full citation is as follows:

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*indicates co-first authors

KAK, AMW, KGN, and ED performed the behavioral and immunohistochemical experiments. JFN provided the cAV-cre. KAK, AMW, and SMF designed the experiments and wrote the manuscript. All authors contributed to data interpretation and manuscript editing.

2.1 Abstract

The medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) are both integral components of the cortico-basal ganglia-thalamic circuitry that regulates addiction-related behaviors. However, the role of afferent inputs from mPFC to NAc in these behaviors is unclear. To address this, we used a Cre-recombinase dependent viral vector approach to express $G_{i/o}$ -coupled DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) selectively in mPFC neurons projecting to the NAc and examined the consequences of attenuating activity of these neurons on the induction of amphetamine sensitization and on drug-taking and drug-seeking during cocaine self-administration. Surprisingly, decreasing mPFC afferent activity to the NAc only transiently reduced locomotor sensitization and had no effect on drug-taking during cocaine self-administration. However, inhibiting corticostriatal afferent activity during sensitization subsequently enhanced conditioned responding. In addition, this manipulation during drug self-administration resulted in slower rates of extinction and increased responding during drug prime-induced reinstatement – an effect that was normalized by inhibiting these corticostriatal afferents immediately prior to the drug prime. These results suggest that dampening cortical control over the NAc during drug exposure may lead to long-term changes in the ability of drugs and associated stimuli to drive behavior, which has important implications for guiding treatments to prevent relapse.

2.2 Introduction

Drug addiction is a debilitating neuropsychiatric disease characterized by a transition from controlled to uncontrollable drug-taking and drug-seeking and a high propensity to relapse, even after prolonged periods of drug abstinence. Chronic drug use produces adaptations within cortico-basal ganglia-thalamic circuitry that are thought to underlie the behaviors that emerge during various stages of addiction (Kalivas & Volkow, 2011; Moussawi et al., 2011; Shiflett & Balleine, 2011a). For example, morphological, electrophysiological and neurochemical changes have been reported in both the striatum (i.e. nucleus accumbens (NAc) and dorsal striatum), which is the central interface of this circuit, and the prefrontal cortex (PFC), which sends a strong glutamatergic input into the striatum (Berke & Hyman, 2000; Lüscher & Malenka, 2011; Nestler, 2001; Russo et al., 2010; Schmidt & Pierce, 2010; Steketee & Kalivas, 2011). Although it has been postulated that a progressive reduction in PFC control over the striatum underlies many of the behaviors that contribute to addiction (Feil et al., 2010; Goldstein & Volkow, 2011; Kalivas et al., 2005; Steketee & Kalivas, 2011), until recently there has been little direct evidence for this, in part because the high degree of neuronal interconnectivity between the cortex and other regions of the cortico-basal ganglia-thalamic circuit has made these studies difficult to conduct. In addition, the striatum receives innervation from multiple glutamatergic sources (thalamus, amygdala and hippocampus) along with the cortex (McGeorge & Faull, 1989; Wall et al., 2013), so studies using traditional approaches, such as lesions or pharmacological blockade, have been unable to isolate which projections are critical for modulating striatal function in addiction-related behaviors. Thus, elucidating how PFC afferents to striatum in

particular regulate behaviors associated with psychostimulant use is crucial for our understanding of the neural substrates that mark a transition to addiction as well as those that underlie relapse.

To investigate this, we used a novel Cre-recombinase (Cre-) dependent, intersectional viral vector approach to express $G_{i/o}$ -DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) selectively in medial PFC (mPFC) afferents to NAc. DREADDs are only activated by clozapine-N-oxide (CNO); thus, this strategy allows for the transient and targeted activation of $G_{i/o}$ -coupled signaling cascades in these neurons (Rogan & Roth, 2011). Activation of $G_{i/o}$ -DREADDs by CNO decreases neuronal activity primarily through a reduction in cAMP levels as well as activation of G-protein coupled inwardly-rectifying potassium (GIRK) channels, which results in membrane hyperpolarization and inhibition of neuronal firing (Armbruster et al., 2007; Ferguson et al., 2010; Sternson & Roth, 2014). Accordingly, these tools were used to examine how transiently decreasing activity of mPFC neurons that project to NAc through recruitment of inhibitory G-protein coupled signaling cascades impacts the induction of psychomotor sensitization to amphetamine as well as the motivation to take drugs using a progressive ratio (PR) schedule of reinforcement in a cocaine self-administration paradigm and the motivation to seek drugs during drug prime-induced reinstatement. We hypothesized that decreasing activity of the cortical projections to NAc would block amphetamine sensitization as well as attenuate drug-taking during cocaine self-administration and drug-seeking during cocaine prime-induced reinstatement.

2.3 Materials and Methods

Experimental strategy. The overall experimental strategy was to use a combinatorial viral vector approach to express transgenes selectively in mPFC afferents to the NAc. This was achieved through injection of Cre-recombinase (Cre)-dependent Adeno-Associated Virus (AAV) vectors into mPFC and a retrogradely transported canine adenovirus (CAV) expressing Cre into the NAc. Three separate experiments were then performed. In Experiment 1, cocaine-induced c-Fos expression was measured in the mPFC and the NAc following activation of $G_{i/o}$ -DREADDs in PFC to determine whether dampening PFC afferent input into the NAc was sufficient to decrease activity in NAc neurons. In separate experiments, the effect of dampening mPFC afferent input into the NAc was examined during the induction of amphetamine sensitization (Experiment 2) and during cocaine self-administration (Experiment 3) to determine whether corticostriatal projections modulate behaviors related to addiction. All experimental procedures were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health (NIH) guidelines.

Viral Vectors. Cre-dependent AAV (serotype 5) vectors driven by the human synapsin promoter and expressing $G_{i/o}$ -DREADDs (AAV-hSyn-DIO-hM4Di-mCherry; hM4Di) or the control GFP (Green Fluorescent Protein; AAV-hSyn-DIO-EGFP; GFP) were constructed by Dr. Bryan Roth and obtained from the University of North Carolina viral vector core (titer of approximately 1×10^9 viral genomes/ml). CAV2-Cre (originally obtained from Dr. Eric Kremer) was prepared in dog kidney (DK/E1-1) cells, purified by sucrose and CsCl gradient centrifugation steps, and resuspended in 1x Hanks Balanced

Saline Solution at a titer of approximately 2.5×10^9 viral genomes/ml as described previously (Kremer et al., 2000).

Experiment 1 (c-Fos). Male Long Evans rats (n=22, Charles River) weighing ~250-300g were pair housed in a temperature- and humidity-controlled vivarium on a 12:12 h light–dark cycle and maintained on ad libitum food and water access. For viral-mediated gene transfer, rats were anesthetized with 2-4% isoflourane (Webster Veterinary Supply) and given meloxicam (0.2 mg/kg, sc) for pain management. Rats were monitored for at least 3 days following surgical procedures. Using standard stereotaxic procedures, 27-gauge stainless steel injectors were placed above the targeted brain regions. Coordinates from bregma (mm) for mPFC: A/P 3.2; M/L ± 1.4 ; D/V -3.5 from skull surface and for NAc: A/P 1.7; M/L ± 1.0 ; DV -6.5. For assessment of cFos in the NAc, 2 ml of GFP was injected in one hemisphere of the mPFC, 2 ml of hM4Di was infused into the contralateral hemisphere of the mPFC and 2 ml of CAV-Cre was infused bilaterally into the NAc over a 10 min period at a flow rate of 0.2 ml/min. Thus, each rat (n=7) had GFP in one hemisphere and hM4Di in the other hemisphere, which allowed for a within-subject design for this experiment. For assessment of cFos in the mPFC and in the basolateral amygdala (BLA) rats received bilateral injections of CAV-Cre into NAc and bilateral injections of GFP (n=7) or hM4Di (n=7) in the mPFC, which allowed for a between-subject design. Accuracy of injection coordinates was confirmed by visualization of GFP or mCherry immunofluorescence in mPFC. Twenty days following viral infusions, rats were transported to a novel test environment and given an injection of CNO (3 mg/kg, ip; obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the NINDs) followed 20 min later by an

injection of cocaine (20 mg/kg, ip). Two hours later rats were euthanized and brains were processed for immunohistochemistry; c-Fos cells were counted in the mPFC, the NAc, and the BLA. In addition, to assess whether the mPFC neurons projecting to the NAc also send collaterals to other brain regions within the corticostriatal circuit, GFP fluorescence was assessed in the NAc, the BLA, and the ventral hippocampus (VH).

Experiment 2 (sensitization). Male Sprague Dawley rats (n=52) underwent viral-mediated gene transfer surgery as described for Experiment 1 except that the surgical coordinates and injection volumes were modified. Coordinates from bregma (mm) for mPFC: A/P 2.8; M/L \pm 0.8; D/V -4.5 from skull surface and for NAc: A/P 1.8; M/L \pm 1.0; DV -7.5 and rats received 1 ml of each virus at each site.

The psychomotor activating effects of amphetamine were measured using locomotor activity boxes (San Diego Instruments). Briefly, at least 14 days following viral infusions rats received four injections of amphetamine (2 mg/kg, ip, Sigma) or vehicle (0.9% saline, ip) over a 7-day treatment period (one injection occurring every other day). Twenty minutes prior to each drug treatment, all rats received an injection of CNO (2 mg/kg, ip). Following injections of amphetamine or vehicle, rats were placed into the locomotor activity boxes where behavior was recorded for 90 min. After a 14-day withdrawal period, all rats underwent a challenge session. First, rats habituated to the locomotor chambers for 30 minutes. Then they received an injection of saline followed by 30 minutes of behavioral testing to assess for a conditioned response. Finally, rats received a low-dose amphetamine challenge (0.5 mg/kg, ip) in the absence of CNO pretreatment followed by 90 min of behavioral testing to assess for the persistence of sensitization. The number of cage crossovers, defined as two consecutive beam

breaks, was used as an index of locomotor activity. Stereotypy ratings were also assessed during testing using an adapted 9-point rating scale (Dougherty & Ellinwood, 1983). Rats were observed by an experimenter blind to the experimental conditions for 30-sec every 5-min during the test sessions and were given a stereotypy rating during each observation (1-asleep; 2-inactive; 3-normal in-place activity; 4-normal, alert, active; 5-hyperactive; 6-slow patterned stereotyped behaviors; 7-fast patterned stereotyped behaviors; 8-restricted stereotyped behaviors; 9-dyskinetic-reactive). There were four groups used in the psychomotor sensitization experiments: hM4Di rats injected with amphetamine (n=13) or saline (n=12) and GFP rats injected with amphetamine (n=13) or saline (n=12). Two rats were excluded from the analysis because virus expression was outside of mPFC.

Experiment 3 (self-administration). Male Long Evans rats (n=35) underwent viral-mediated gene transfer surgery as described in Experiment 1 with rats receiving bilateral injections of either GFP (n=7) or hM4Di (n=15) into the mPFC and bilateral injections of CAV-Cre into the NAc. Following recovery, indwelling jugular catheters were implanted as previously described (Kerstetter et al., 2008). Briefly, catheters were inserted into the right jugular vein and connected to a back-mounted port. Catheters were flushed daily with Timentin antibiotic (20 mg/kg, iv; Butler Schein) and catheter patency was verified periodically with methohexital sodium infusions (10 mg/ml iv; Eli Lilly).

At least 14 days following viral infusions, rats were trained to self-administer cocaine (0.75 mg/kg/infusion in 100 mL of 0.9% sterile saline administered over 4 s; obtained from the National Institute on Drug Abuse) during their light cycle on a Fixed

Interval: 20 s (FI:20) schedule of reinforcement. Each cocaine infusion was paired with a 5 s light and tone stimulus. Self-administration sessions lasted 2-hours and took place 5 days per week in sound-attenuated operant conditioning chambers (Med Associates).

After rats had met self-administration criteria (minimum of 6 training sessions with 10 or more cocaine infusions earned for 3 consecutive sessions), they began PR sessions during which the response requirement to earn a cocaine infusion increased after each infusion earned. The response requirement progression followed that of (Richardson & Roberts, 1996) and was as follows: 1,2,4,6,9,12,15,20,25,32,40,50,62,77,95,118,145,178,219,268,328,402,492,603. PR sessions were limited to 2-h due to the duration of CNO treatment (which was given during the testing phase). Once behavior had stabilized (≤ 2 -step change in the last response requirement completed in a session over 2 consecutive sessions), rats were administered vehicle (6% DMSO in sterile water, ip) 20 min prior to PR testing for three sessions to obtain a baseline response. Rats were then administered CNO (3 mg/kg, ip) 20 min prior to PR testing for three sessions. Following testing, rats underwent three additional PR sessions in the absence of any pretreatment to determine if CNO treatment had any lasting effects on PR responding.

A subset of rats (GFP: n=7; hM4Di: n=5) then underwent extinction of operant responding until criterion was met (a minimum of 7 sessions with at least 2 consecutive sessions of ≤ 25 active lever responses). During the 2-h extinction sessions, levers were extended but responding did not have any programmed consequences (i.e., no infusions or light/tone cue were given). Rats then received vehicle treatment immediately prior to being placed into the operant chamber to establish a baseline of

responding. Rats then underwent two cocaine prime-induced reinstatement tests (10 mg/kg, ip) with either DMSO or CNO (3 mg/kg, ip) given 20 min prior to the cocaine injections. Cocaine injections were given immediately prior to reinstatement testing, and levers were extended for these sessions but responding did not have any programmed consequences. These pretreatments were given in a counter-balanced fashion and rats received additional extinction training sessions following the first reinstatement test (see Figure 4 for illustration of experimental design). Thirteen rats were excluded from the experiments because 4 rats had injection sites outside of the mPFC, 7 rats failed to acquire cocaine self-administration and 2 rats were outliers from the behavioral data set (>2 standard deviations away from the mean).

Immunohistochemistry. Rats were anesthetized with Beuthanasia-D (Schering-Plough) and perfused transcardially with 1x PBS (pH 7.4), followed by 4% paraformaldehyde (PFA). Brains were extracted, post-fixed in 4% PFA overnight and stored in 1x PBS. Floating sections (40-60 μ m) were washed in 0.5% Triton-X/PBS for 10 min, blocked in 5% normal goat serum or 7.5% normal donkey serum (NS)-0.25% Triton-X/PBS for 2 h and incubated in 2.5-5% NS-0.25% Triton-X/PBS containing antibodies to GFP (1:400, Millipore), mCherry (1:400, Clontech) or c-Fos (1:400, Santa Cruz) with gentle agitation for 24-72 h. Next, sections were rinsed 4 times in PBS and incubated in species-appropriate Alexa 488 (green), Alexa 568 (red) or Alexa 647 (far red)-conjugated secondary antibodies (1:400, Invitrogen) for 2 h. Sections were washed 2 times in PBS, mounted on slides and cover-slipped with Vectashield mounting medium with DAPI (Vectorlabs). Z-stack images were captured with a Zeiss confocal microscope and compressed into a single plane prior to quantification. c-Fos+ cells in

the mPFC, NAc and BLA were counted and averaged across 3-4 sections for each rat using ImageJ software (NIH).

Data analysis. All analyses consisted of planned (a priori) comparisons. Group differences in crossovers, stereotypy ratings, active and inactive lever presses, and number of earned infusions were tested using two-way analysis of variance (ANOVA) with repeated measures when applicable, followed by Bonferroni's post-hoc tests. Differences in the number of c-Fos+ cells were tested using a paired t-test for the NAc and unpaired t-tests for the mPFC and the BLA. For all comparisons, $\alpha \leq 0.05$. Data is graphed as mean \pm SEM.

2.4 Results

The intersectional viral vector approach produces transgene expression primarily in mPFC neurons projecting to the NAc. A Cre-dependent intersectional vector approach was used to express hM4Di receptors in mPFC neurons projecting to the NAc in order to selectively and transiently decrease activity of these afferents (Fig. 2.1a,b). Viral expression in the mPFC was largely confined to the cingulate and prelimbic regions (Fig. 2.1b). Although transgene expression was induced by retrograde infection of Cre from NAc neurons, it is possible that the DREADD-expressing mPFC neurons send collaterals to other regions. In order to confirm specificity of the intersectional approach, we examined fluorescence expression in mPFC terminals in the NAc, as well as the BLA and the VH; two output regions of the mPFC that also send glutamatergic inputs into the NAc. We observed a strong amount of fluorescence in the NAc (Fig.

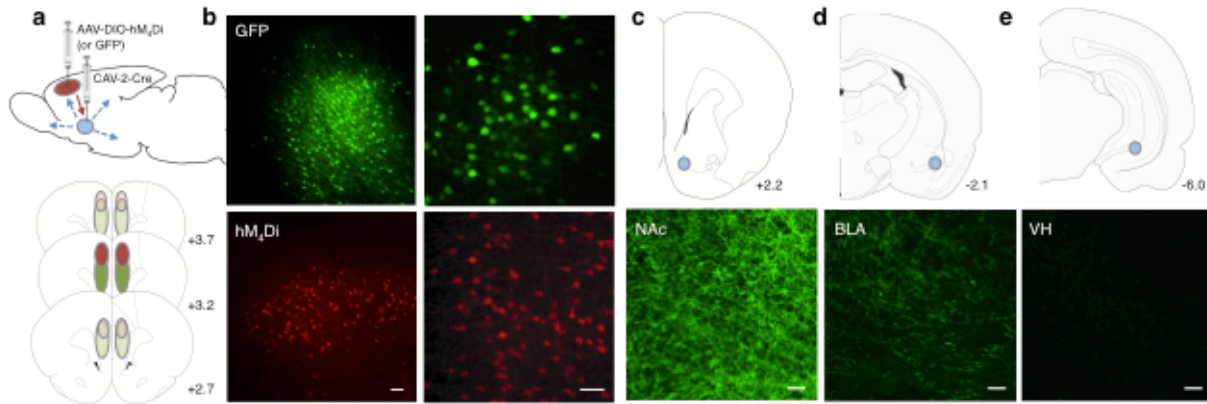


Figure 2.1. Viral vector expression. (a) Top: Illustration of the intersectoral viral vector approach: Cre-recombinase (Cre) dependent AAV vectors (hM₄Di or GFP) were injected into the medial prefrontal cortex (mPFC) and the retrograde CAV-Cre virus was injected into the nucleus accumbens (NAc). Bottom: Viral spread is depicted in red for the self-administration experiments and in green for the sensitization experiments. Dark shading indicates areas of robust expression and light shading indicates areas with weaker expression. (b) Representative sections of GFP (top) and mCherry-tagged hM₄Di (bottom) immunofluorescence in PFC 14 days following viral infusions. (c-e) Representative sections of GFP immunofluorescence in NAc (c), basolateral amygdala (BLA) (d) and ventral hippocampus (VH) 8 weeks following viral infusions. Scale bars, 40 μm.

2.1c), with some fluorescence in the BLA (Fig. 2.1d) and no fluorescence in the VH (Fig. 2.1e), suggesting that DREADD receptor expression was occurring primarily in mPFC neurons that were projecting selectively to the NAc.

Decreasing activity of mPFC afferents to NAc reduces cocaine-induced c-Fos in both PFC and NAc neurons. It is well established that activation of hM₄Di receptors by CNO decreases neuronal activity primarily through a reduction in cAMP levels as well as activation of GIRK channels (Armbruster et al., 2007; Ferguson et al., 2010; Sternson & Roth, 2014). This effect has been observed in glutamatergic pyramidal neurons of the cortex, which is the population of neurons that expressed hM₄Di receptors in the present set of experiments (Kätzel et al., 2014; Kozorovitskiy et al., 2012; Robinson et al., 2014). Using cocaine-induced stimulation of the immediate early gene c-Fos as a marker of neuronal activity, we found that consistent with these

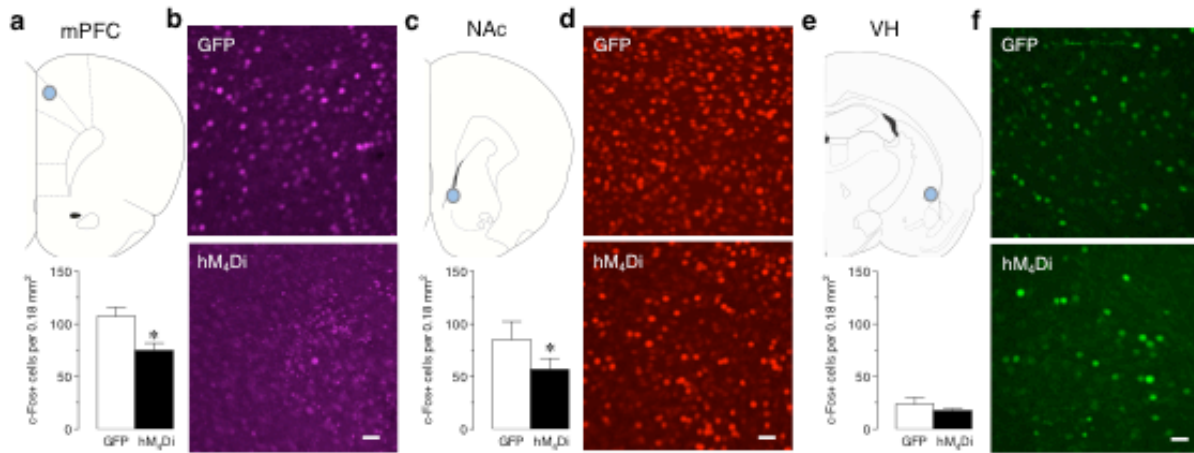


Figure 2.2. c-Fos expression. (a,c,e) CNO-mediated activation of hM₄Di-expressing mPFC neurons significantly reduces the number of cocaine-induced c-Fos⁺ cells in PFC (a, **P* < 0.05 versus GFP group, N=6-10) and NAc (c, **P* < 0.05 versus GFP hemisphere, N=7) but not in BLA (e, *P* = 0.38 versus GFP group, N=7-8) (b,d,f) Representative sections of Fos immunohistochemistry in mPFC (b), NAc (d) and BLA (f) are shown from GFP (top) and hM₄Di sections (bottom). Scale bars, 40 μm

studies, activation of hM₄Di receptors in mPFC significantly decreased the number of cocaine-evoked c-Fos⁺ cells in mPFC by ~30% compared to the GFP controls (Fig. 2.2a,b; $t_{14} = 2.68$ *P* = 0.02).

In order to extend these findings, we examined whether the manipulation in mPFC was sufficient to alter activity of the striatal neurons receiving mPFC input. Indeed, we found that activation of hM₄Di receptors in mPFC significantly decreased the number of cocaine-evoked c-Fos⁺ cells in NAc by ~32% compared to the GFP control hemisphere (Fig. 2.2c,d; $t_6 = 3.56$, *P* = 0.01), suggesting that decreasing activity of mPFC projections to NAc reduces the neuronal activity of its downstream targets. In contrast, CNO-induced activation of hM₄Di receptors in mPFC had no effect on cocaine-evoked c-Fos⁺ cells in the BLA (Fig. 2.2e,f; $t_{13} = 0.9$, *P* = 0.38) suggesting that the manipulation was selective for altering activity in mPFC to NAc neurons.

Decreasing activity of mPFC afferents to NAc alters the induction of amphetamine sensitization. We used the intersectional DREADD viral vector approach

to examine whether decreasing activity of mPFC afferents to NAc during amphetamine administration would be sufficient to block the induction of this progressive and persistent form of drug-induced behavioral plasticity. We found that CNO-induced activation of hM4Di receptors had no effect on locomotor activity following saline injections (Fig. 2.3a; main effect of virus $F_{1,22}=4.97$, $P=0.04$, main effect of session $F_{3,66}=6.12$, $P=0.001$, no interaction between session and virus $F_{3,66}=1.35$, $P=0.27$), suggesting a lack of effect of this manipulation on baseline activity. Although both locomotor and stereotyped responses to amphetamine increased over sessions in GFP and hM4Di rats (Fig 2.3a,b; locomotion: main effect of virus $F_{1,24}=6.18$, $P=0.02$, main effect of session $F_{3,72}=15.39$, $P<0.0001$, no interaction between session and virus $F_{3,72}=1.15$, $P=0.34$; stereotypy: main effect of session, $F_{3,72}=99.3$, $P<0.0001$, no effect of virus $F_{1,24}=0.84$, $P=0.37$, no main effect of session and virus, $F_{3,72}=0.84$, $P=0.48$), hM4Di rats had significantly reduced levels of locomotor activity during the last session compared to GFP controls ($P < 0.05$), suggesting that dampening activity of mPFC afferents to NAc attenuates the induction of locomotor sensitization.

Following two weeks of withdrawal, all rats underwent a challenge session. This was performed in the absence of CNO in order to assess whether decreasing activity of mPFC afferents to NAc during the induction of amphetamine sensitization has lasting effects. All rats underwent 30 minutes of habituation in the locomotor chambers, and there was no effect of the DREADD manipulation on locomotor activity during this phase (data not shown). Next rats received an injection of saline to assess whether decreasing mPFC afferent activity during the induction phase altered conditioned responding to the injection procedure. Interestingly, hM4Di rats that had previously received

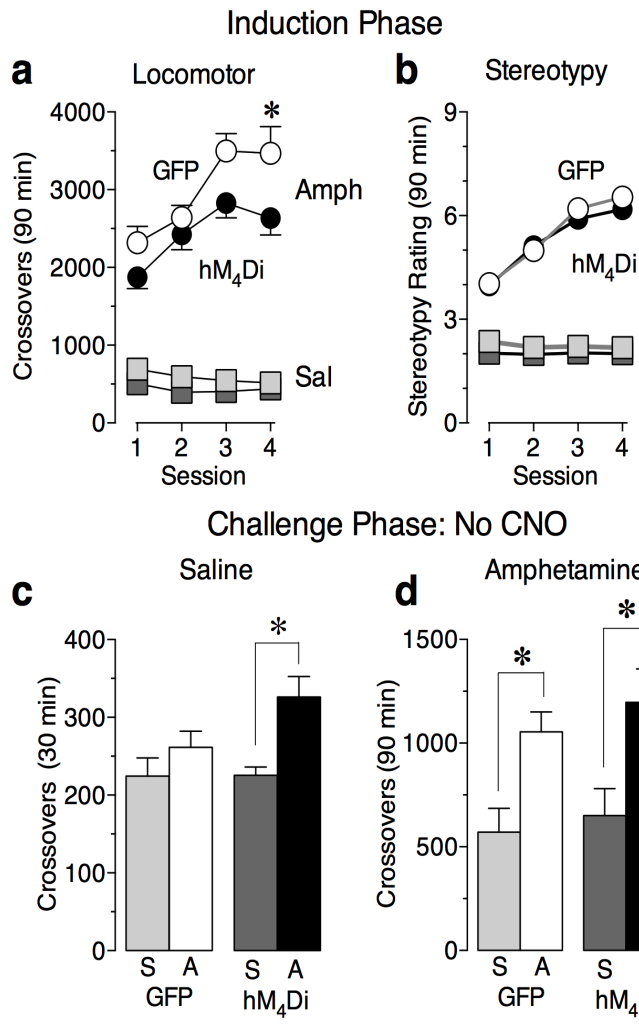


Figure 2.3. Amphetamine sensitization. Decreasing activity of mPFC afferents to NAc alters the development of amphetamine sensitization. CNO-mediated activation of hM₄Di in corticostriatal afferents significantly decreased locomotor sensitization to amphetamine as measured by crossovers (**a**, * $P < 0.05$ versus GFP group), but had no effect on stereotypy ratings (**b**). Decreasing activity of mPFC afferents to NAc during the induction phase of sensitization significantly increased conditioned responding as measured by crossovers during a saline challenge (**a**, * $P < 0.05$ versus hM₄Di group given saline during the induction phase), but had no effect on the persistence of locomotor sensitization as measured by crossovers during a low-dose amphetamine challenge (0.5 mg/kg, *ip*) in the absence of CNO pretreatment (**d**, * $P < 0.05$ versus respective control groups given saline during the induction phase). Data represent mean \pm SEM. Black symbols/shading (hM₄Di) and white symbols/shading (GFP) represent groups that received amphetamine treatment during the induction phase. Dark grey symbols/shading (hM₄Di) and light grey symbols/shading (GFP) represent groups that received saline treatment during the induction phase. S: saline during the induction phase, A: amphetamine during the induction phase. N=11-17/group.

amphetamine showed enhanced locomotor activity during this test compared to the hM₄Di rats that had received saline, an effect not observed in the GFP groups (Fig. 2.3c; main effect of drug pretreatment $F_{1,46}=2.25$, $P=0.002$) suggesting that only hM₄Di rats that received amphetamine treatment developed a conditioned response to the injection procedures associated with the drug. Finally, all rats received an injection of a low dose of amphetamine (0.5 mg/kg, *ip*). Both GFP and hM₄Di rats that had received amphetamine during the induction phase showed similar levels of enhanced locomotor activity relative to the rats that had received saline during the induction phase (Fig. 2.3d; main effect of drug pretreatment $F_{1,46}=16.03$, $P=0.0002$, no main effect of

virus $F_{1,46}=0.74$, $P=0.39$, no interaction between drug pretreatment and virus $F_{1,42}=0.06$, $P=0.81$), indicating that the level of sensitization during the challenge phase was similar across groups.

Decreasing activity of mPFC afferents to NAc has no effect on motivation for taking drugs. To determine whether decreasing activity of mPFC afferents to NAc alters drug-taking in a cocaine self-administration paradigm, rats were trained to self-administer cocaine first on a FI:20 schedule and then on a PR schedule of reinforcement until stable baselines were achieved (Fig. 2.4). During the last three PR training sessions prior to testing, both GFP and hM4Di rats made significantly more active lever responses compared to inactive lever responses (Fig. 2.4a; main effect of lever, hM4Di: $F_{1,28} = 51.70$, $P < 0.0001$; GFP: $F_{1,12} = 50.73$, $P < 0.0001$) but there were no differences in lever responding across sessions (Fig. 2.4a; no main effect of session, hM4Di: $F_{2,56} = 1.57$, $P = 0.38$; GFP: $F_{2,24} = 0.37$, $P = 0.70$ and no interaction between lever and session, hM4Di: $F_{2,56} = 1.00$, $P = 0.38$; GFP: $F_{2,24} = 0.47$, $P = 0.63$). In addition, there were no differences in either active lever responses (Fig. 2.4a; no main effect of virus $F_{1,20} = 0.20$, $P = 0.66$, no main effect of session $F_{2,40} = 0.03$, $P = 0.97$ and no interaction between virus and session $F_{2,40} = 1.42$, $P = 0.25$) or number of earned infusions completed (Fig. 2.4b; no main effect of virus $F_{1,20} = 0.14$, $P = 0.71$, no main effect of session $F_{2,40} = 0.30$, $P = 0.74$ and no interaction between virus and session $F_{2,40} = 0.59$, $P = 0.56$) between GFP and hM4Di rats suggesting that both groups had acquired the same stable levels of cocaine self-administration. Both groups then received vehicle pretreatment for three sessions, followed by CNO pretreatment for three sessions and three additional baseline sessions (data averaged across sessions).

Schedule	FI:20	Progressive Ratio			Extinction	Reinstatement			
Treatment	--	--	V	C	--	SAL	V + COC ^x	Ext	C + COC ^x
Sessions	7-10	5-11	3	3	3	1	1	3	1

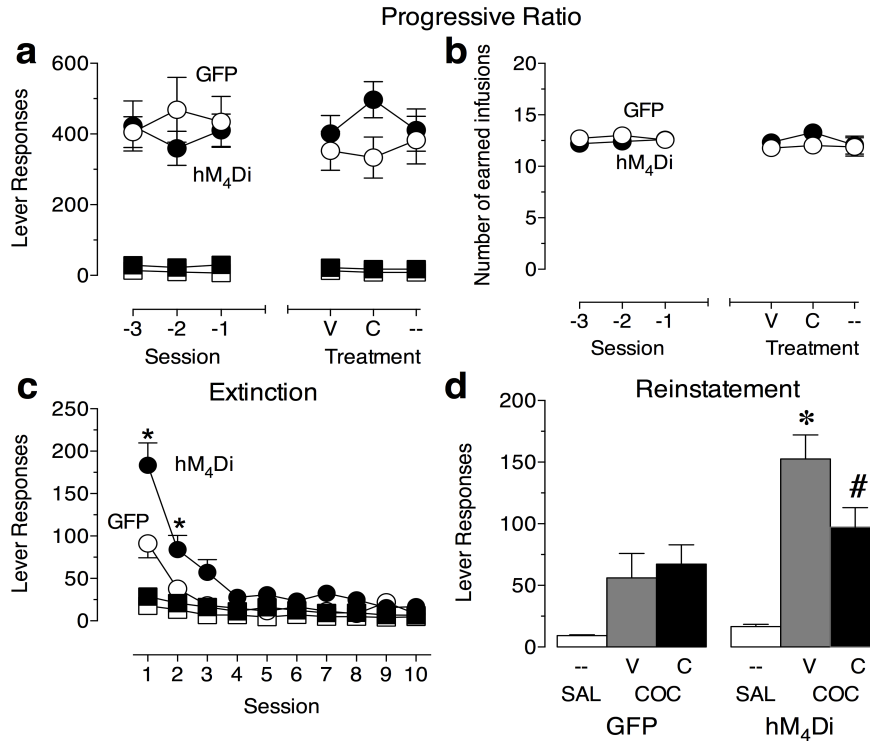


Figure 2.4. Cocaine self-administration. Decreasing activity in mPFC afferents to NAc modulates drug-seeking. There were no differences between groups in active (circles) and inactive (squares) lever responses (**a**, left panel) and number of earned infusions (**b**, left panel) during baseline progressive ratio (PR) cocaine self-administration (last 3 sessions prior to testing). CNO-mediated activation of hM₄Di in corticostriatal afferents had no effect on lever responses (**a**, right panel) or number of earned infusions (**b**, right panel). Rats that had hM₄Di activation during PR showed a significant increase in the number of active lever responses during the first 2 sessions of extinction training. (**c**, * $P < 0.05$ versus GFP group) as well as following a cocaine prime (**d**, * $P < 0.05$ versus GFP group). CNO pretreatment prior to a cocaine prime significantly attenuated active lever responding only in the hM₄Di group (**d**, # $P < 0.05$ versus V-treated hM₄Di group). Data represent mean \pm SEM. Black symbols in line graphs represent the hM₄Di group and white symbols represent the GFP controls. ^xNote that pretreatments (V or C) were counterbalanced across rats during reinstatement tests. SAL: saline injection. COC: cocaine injection. V: vehicle pretreatment. C: CNO pretreatment. N=5-7/group.

There were no differences between groups in active lever responses (Fig. 2.4a; no main effect of virus $F_{1,20} = 1.03$, $P = 0.32$, no main effect of drug pretreatment $F_{2,40} = 0.53$, $P = 0.60$ and no interaction between virus and drug pretreatment $F_{2,40} = 1.90$, $P = 0.16$) or number of earned infusions (Fig. 2.4b; no main effect of virus $F_{1,20} = 0.42$, $P = 0.53$, no main effect of drug pretreatment $F_{2,40} = 1.07$, $P = 0.35$, and no interaction

between virus and drug pretreatment $F_{2,40} = 0.60$, $P = 0.55$) across test sessions, suggesting that transiently decreasing corticostriatal afferent activity has no effect on drug taking.

Decreasing activity of mPFC afferents to NAc alters motivation for seeking drugs.

To test whether decreasing activity of mPFC afferents to NAc alters drug-seeking during drug-primed reinstatement, a subset of rats went through extinction training. Unexpectedly, hM4Di rats that had received CNO treatment during PR testing made significantly more active lever responses during the first two days of extinction training compared to the GFP controls (Fig. 2.4c; main effect of virus $F_{1,10} = 12.90$, $P = 0.005$, main effect of session $F_{6,60} = 50.07$, $P < 0.0001$ and interaction between virus and session $F_{6,60} = 5.76$, $P < 0.0001$), suggesting an impairment in their extinction learning. In addition, although both groups reached extinction criteria by 10 sessions, hM4Di rats made significantly more active lever responses following a priming injection of cocaine compared to the GFP controls (Fig. 2.4d; main effect of virus $F_{1,10} = 2.67$, $P = 0.03$, main effect of drug treatment $F_{2,20} = 38.96$, $P < 0.0001$ and interaction between virus and drug treatment $F_{2,20} = 9.19$, $P = 0.002$), indicating a greater level of reinstatement. Interestingly, CNO treatment immediately prior to the cocaine prime significantly attenuated reinstatement in the hM4Di rats (Fig. 2.4d; main effect of virus $F_{1,10} = 9.56$, $P = 0.01$, main effect of pretreatment $F_{2,20} = 38.96$, $P < 0.0001$ and interaction between virus and pretreatment $F_{2,20} = 9.19$, $P = 0.002$). These effects were not due to indiscriminate alterations in activity, as inactive lever responses did not differ between groups during extinction training (Fig. 2.4c; no main effect of virus $F_{1,10} = 3.70$, $P = 0.08$ and no interaction between virus and session $F_{6,60} = 0.38$, $P = 0.89$) or during

cocaine-prime induced reinstatement (no main effect of virus $F_{1,10} = 0.29$, $P = 0.60$, no main effect of drug pretreatment $F_{1,10} = 3.94$, $P = 0.08$, and no interaction between virus and drug pretreatment $F_{1,10} = 1.26$, $P = 0.29$), and CNO had no effect on cocaine prime-induced reinstatement in the GFP controls (Fig. 4d; $P = 0.80$).

2.5 Discussion

We used a Cre-dependent intersectional viral vector approach to express $G_{i/o}$ -DREADDs in mPFC neurons that project to the NAc. Consistent with the known mechanism of hM4Di receptor activation, which is to reduce excitability of the cells expressing the $G_{i/o}$ -DREADDs (Armbruster et al., 2007; Ferguson et al., 2010; Sternson & Roth, 2014), we found that CNO-induced receptor activation reduced cocaine-evoked c-Fos in the region of viral expression. In order to assess the specificity of this approach, we compared immunofluorescence in GFP-expressing rats in three terminal regions of the mPFC - the NAc, the BLA and the VH. Although the densest amount of fluorescence was observed in the NAc, fluorescence was also apparent in the BLA, suggesting that the corticostriatal neurons that were expressing $G_{i/o}$ -DREADDs may send axon collaterals to other regions. However, it is also possible that the signal in the BLA was simply due to axons passing through the region. In line with this idea, we found that activation of hM4Di receptors in mPFC attenuated cocaine-evoked c-Fos in NAc neurons but not in BLA neurons. Cocaine-induced activation of c-Fos in striatal cells is modulated by glutamate (Harlan & Garcia, 1998); thus, it is likely that the reduction in c-Fos that we observed following hM4Di receptor activation of corticostriatal neurons was due to a decrease in glutamatergic stimulation of the NAc neurons. In

addition, these results indicate that the intersectional approach was selective for modulating corticostriatal afferent activity.

Next, we examined the consequences of transiently decreasing activity in corticostriatal neurons during amphetamine administration on psychomotor sensitization. Given that previous work has demonstrated a role for the mPFC in regulating sensitization (Kalivas et al., 2005; Steketee & Kalivas, 2011; Tzschentke & Schmidt, 2003; Vanderschuren & Kalivas, 2000; Wolf, 1998), and many of the sensitization-related neurobiological changes that occur in the NAc are glutamate dependent (Kalivas et al., 2005; Steketee & Kalivas, 2011), we hypothesized that selectively dampening mPFC activity to the NAc would decrease the induction of sensitization. Consistent with this hypothesis, decreasing activity of mPFC afferents to NAc during repeated amphetamine administration attenuated locomotor sensitization without altering stereotyped responses, which are thought to be regulated by the dorsal striatum (Solomon & Staton, 1982). Unexpectedly, locomotor sensitization appeared equivalent between groups during the challenge phase. There are several possible explanations that could account for these results. One, the observed effects during the induction phase could be due to a decrease in the expression of sensitization; this is unlikely to be the case as differences in the level of sensitization were only observed during the last treatment session. Two, it is possible that our manipulation initially decreased the induction of sensitization, but the underlying neurobiological changes that occur in corticostriatal circuits during withdrawal permitted sensitization to develop normally over the long-term. And three, it may be that decreasing mPFC afferent activity to the NAc did, in fact, permanently disrupt the induction of sensitization, and the

response during the amphetamine challenge was a reflection of other processes that were altered during the DREADD manipulation, such as an increase in conditioned responding. In support of this last idea, we found that attenuating activity in cortical projections to NAc during the induction of amphetamine sensitization resulted in the development of a conditioned response to the injection procedure as measured during the saline challenge - an effect not seen in the controls.

Nonetheless, these results suggest that direct modulation of NAc activity by the mPFC is unlikely to be a critical node in the modulation of behavioral sensitization. Instead, it is likely that the mPFC exerts its effects on NAc indirectly through regulation of other NAc inputs, such as VTA, to modulate sensitization, and other sources of glutamate (i.e., amygdala, hippocampus or thalamus) must be responsible for direct modulation of the NAc. Consistent with this idea, it was recently demonstrated that optical inhibition of VH inputs into the NAc shell was sufficient to reduce the development of locomotor sensitization to cocaine (Britt et al., 2012). In addition, decreasing activity of the BLA via *Gi/o*-DREADDs not only attenuated the development of cocaine sensitization but also blocked cocaine-induced increases in the frequency of miniature excitatory post-synaptic currents in NAc neurons, suggesting that NAc activity is directly modulated by amygdala afferents (MacAskill et al., 2014). However, it should be noted that in the present set of experiments DREADD receptors were primarily expressed in the cingulate and prelimbic regions of the mPFC; thus we cannot rule out that targeting a larger region of the PFC and/or more medial aspects could lead to a different behavioral outcome. This is unlikely to be the case, however, as previous work

has demonstrated that lesions of or pharmacological manipulations to this region of mPFC are sufficient to modulate sensitization (Tzschentke & Schmidt, 2000).

Finally, we examined the consequences of transiently decreasing activity in corticostriatal neurons on drug-taking during cocaine self-administration and on drug-prime induced reinstatement following extinction. Although this manipulation had no effect on on-going drug use during self-administration, inhibiting mPFC afferent activity to the NAc immediately prior to the drug-prime decreased reinstatement. This finding is consistent with recent studies that found that optogenetic inhibition of prelimbic PFC fibers in the NAc core also blocks both cocaine prime-induced reinstatement and cocaine-plus-cue induced reinstatement (Stefanik et al., 2013; 2015). In addition, it is consistent with recent work demonstrating that reversing silent synapse remodeling (which occurred during withdrawal from cocaine self-administration) in the NAc core by optical stimulation of PFC inputs inhibited cue-induced cocaine seeking (Ma et al., 2014). Finally, these results are in-line with the idea that heightened PFC activation underlies the enhanced responsiveness seen to drugs and drug-related cues during relapse (Feil et al., 2010; Goldstein & Volkow, 2011).

Although decreasing cortical activity to NAc neurons did not alter drug-taking behavior during cocaine self-administration, it did have a large impact on subsequent responsiveness to the environmental stimuli associated with drug administration and to the drug itself. Specifically, decreasing activity in these neurons during cocaine self-administration led to a slower rate of extinction and an increase in active lever pressing during drug prime-induced reinstatement compared to controls. Together with the enhanced conditioned response seen following amphetamine sensitization, these

results suggest that cortical inputs into NAc may be modulating the strength of associations between drugs and the circumstances surrounding drug administration (i.e., 'set and setting') and are consistent with imaging studies in human addicts as well as in preclinical animal models which have found that, relative to controls, extensive psychostimulant use results in a hypoactive PFC at baseline but heightened PFC activation to both the drug and drug-related cues (Goldstein & Volkow, 2011).

Nonetheless, it is perhaps counterintuitive that dampening mPFC afferent activity to NAc during initial drug use would subsequently lead to the development of a conditioned response, as well as the delayed extinction and enhanced responding during drug prime-induced reinstatement (all of which occurred in the absence of DREADD receptor activation). However, although much work has focused on the brain regions and circuits that regulate how drug-associated stimuli drive reinstatement of cocaine-seeking behavior (Everitt & Robbins, 2005; LaLumiere et al., 2012; Marchant et al., 2015), surprisingly little is known about the neural mechanisms that underlie how drugs initially change the incentive value of the stimuli that become associated with drug use. Our results indicate that increasing $G_{i/o}$ -signaling in corticostriatal neurons could help drive the strength of these associative processes. In addition to reducing cAMP activity and activating GIRK channels (Sternson & Roth, 2014), induction of $G_{i/o}$ cascades produces a slow and sustained increase in ERK/MAPK (extracellular signal-regulated kinase) signaling through a β -arrestin mediated pathway (Reiter & Lefkowitz, 2006; Wettschureck, 2005). ERK/MAPK signaling cascades are important modulators of long-term alterations in neuronal plasticity and memory formation (Giovannini, 2006; Sweatt, 2004), and it is of note that the changes in responsiveness to the stimuli

associated with drug use took a period of time to develop, as they were not evident during the induction of sensitization (which appeared blunted) or in the three baseline self-administration sessions that followed CNO treatment. Thus, β -arrestin mediated recruitment of ERK/MAPK cascades during drug use is one possible mechanism that could facilitate the strengthening of associations between the drug and the stimuli. Nonetheless, the striatum receives innervation from multiple glutamatergic sources (thalamus, amygdala and hippocampus) along with the cortex (McGeorge & Faull, 1989; Wall et al., 2013) and it is not yet known whether these inputs work in concert or in opposition to regulate NAc neurons and subsequent behavioral output. Thus, it is also possible that dampening mPFC activity allowed for the information that is carried from these other inputs to have a larger impact on NAc neuron function, thereby facilitating the development of associations between the drug and the 'set and setting' surrounding drug use.

In summary, this work helps to elucidate how mPFC afferents to NAc, in particular, regulate addiction-related behaviors and govern the processes that contribute to relapse. Our results suggest that rather than modulating the maintenance of ongoing behaviors as previously thought, these afferent connections from mPFC to NAc may instead be key for shaping the associations between drugs and the stimuli surrounding drug use, as well as in reinstatement of drug-seeking behaviors. Given that relapse to drugs following exposure to drug-associated stimuli is one of the most insidious facets of addiction, particularly because it is such a persistent phenomenon (Frawley & Smith, 1992; Jones et al., 2003), this work has important clinical implications as it suggests that the mPFC to NAc input would be a promising target for therapeutic intervention.

Chapter 3

Midline thalamic nuclei and thalamostriatal neurons regulate cue-induced and cocaine-primed reinstatement of drug-seeking

*This chapter is currently under preparation for publication. Amanda M. Wunsch, Lindsay M. Yager, Elizabeth Donckels, Calvin Le, John F. Neumaier, and Susan M. Ferguson will be included as co-authors. AMW, LMY, and SMF designed the experiments. AMW, LMY, ED, and CL performed experiments. CL helped with virus localization. AMW and SMF will write the manuscript, and all authors will provide editorial feedback.

3.1 Abstract

Addiction is a debilitating neuropsychiatric illness that produces profound alterations in neuronal function within the corticomesolimbic circuit. Despite advances in understanding the brain circuitry associated with addiction, rates of relapse remain incredibly high. Alterations in glutamatergic signaling within the nucleus accumbens (NAc) are thought to be particularly important in regulating relapse, and inputs originating in prefrontal cortex (PFC), amygdala (AMYG), and hippocampus (HIPPO) can regulate distinct aspects of addiction. Surprisingly, although projection neurons originating in midline thalamic nuclei (MTN) such as paraventricular nucleus of thalamus (PVT) and intermediodorsal (IMD), centromedian (CM), and mediodorsal (MD) nuclei provide the second largest source of glutamate into the NAc, their role in relapse behavior has not yet been clearly identified. In order to define the role of MTN in the reinstatement of cocaine-seeking, we expressed inhibitory, $G_{i/o}$ -coupled DREADDs (hM4Di) in neurons of these nuclei. We found that reducing activity of MTN attenuated both cue-induced and drug-primed reinstatement of cocaine-seeking. Further characterization of hM4Di expression revealed hM4Di in axons and/or terminals within NAc, PFC, and basolateral AMYG. Thus, our manipulation in MTN likely had effects in downstream structures that differentially regulate reinstatement of cocaine-seeking. Because NAc regulates relapse behaviors, we utilized a combinatorial viral and chemogenetic approach to selectively modulate the activity of MTN neurons projecting to NAc (MTN-NAc). Rats received bilateral injections of a retrogradely transported canine adenovirus expressing Cre-recombinase (Cre) into NAc, and an adeno-associated virus expressing Cre-dependent, hM4Di into MTN. Interestingly, we found

that attenuating activity of anterior MTN-NAc neurons enhanced cue-induced reinstatement and attenuated cocaine-primed reinstatement but this same manipulation in posterior MTN-NAc had no effect. These results reveal a novel, yet complex, contribution of $G_{i/o}$ -signaling cascades in MTN-NAc neurons in models of relapse.

3.2 Introduction

Addiction is a neuropsychiatric disorder characterized by cycles of drug-seeking, drug-taking, and abstinence. It is both costly and debilitating with respect to the profound medical and interpersonal consequences for the addict, as well as large social and economic burdens. Unfortunately, due to the persistent nature of addiction, relapse rates remain high, and we currently lack effective treatment options to prevent relapse. The corticomesolimbic system has been identified as the major locus within the brain for the myriad of molecular and cellular adaptations that underlie addiction (Koob & Volkow, 2009; Sesack & Grace, 2009; Steketee & Kalivas, 2011). However, it is a highly interconnected network, and the dearth of treatment success may be due in part to an incomplete understanding of how this complex circuit regulates addiction.

The striatum is a heterogeneous structure within the corticomesolimbic circuit that integrates information from a variety of inputs to regulate responses to drugs of abuse (Kalivas & Volkow, 2011; Sesack & Grace, 2009; Shiflett & Balleine, 2011b). Morphological, physiological, and electrochemical changes have been reported in the nucleus accumbens (NAc) that are thought to underlie the vulnerability to relapse in addicts. In particular, long-lasting changes in glutamate-dependent plasticity within NAc medium spiny neurons leads to an enhancement in glutamatergic signaling that can

contribute to relapse (Berke & Hyman, 2000; Lüscher & Malenka, 2011; Nestler, 2001; Russo et al., 2010; H. D. Schmidt & Pierce, 2010; Steketee & Kalivas, 2011). The NAc receives glutamatergic input from a variety of sources, including the PFC, MTN, AMYG, and HIPP (McGeorge & Faull, 1989; Wall et al., 2013). Recent optogenetic and chemogenetic studies have demonstrated that PFC, AMYG, and HIPP inputs into the NAc regulate distinct aspects of relapse (for review see Yager et al., 2015). For example, optogenetic and chemogenetic studies have shown that inhibition of PFC afferents to NAc attenuated both cocaine-primed and cue+cocaine-primed reinstatement of drug seeking (Kerstetter et al., 2015; Stefanik et al., 2015; 2012) whereas these same neurons can both inhibit and potentiate incubation of drug craving (Ma et al., 2014; Pascoli et al., 2014). AMGY neurons projecting to NAc have been shown to regulate conditioned responses to cues associated with drug-taking and drug-craving (Lee et al., 2013; Stefanik, 2014). Finally, applying an optogenetic LTD stimulation protocol to ventral HIPP neurons projecting to NAc non-discriminatively increased lever pressing during cue-induced reinstatement protocol, suggesting these inputs may regulate appropriate contextual responses associated with drug-craving (Pascoli et al., 2014).

Given that midline and intralaminar nuclei (MTN) are a dense source of striatal glutamate, synapse onto both direct and indirect pathway medium spiny neurons and regulate dopamine release from terminals within the NAc (Frassoni et al., 1997; Lei et al., 2013; Parsons, Li, & Kirouac, 2006; Wall et al., 2013), it is surprising that relatively few studies have explored their role in rodent models of reinstatement of cocaine-seeking (Haight & Flagel, 2014; James & Dayas, 2013). The MTN include

paraventricular (PVT), intermediodorsal (IM), mediodorsal (MD), reunions, and parafasicular nuclei whereas intralaminar nuclei are comprised of centromedian (CM), centrolateral, and parateneal nuclei (Van der Werf et al., 2002). While MTN can be split into discrete clusters of nuclei based on their afferents and efferents, there may be functional homogeneity across all MTN (Berendse & Groenewegen, 1990; Van der Werf et al., 2002). In general, MTN afferents originate in subcortical regions involved in arousal and visceral sensation, such as hypothalamus, dorsal raphe, locus coeruleus, periaqueductal gray, and reticular activating system (Van der Werf et al., 2002). In addition MTN receive input from limbic structures involved in the regulation of emotion and motivation, such as PFC and AMYG (Hsu & Price, 2009; Li & Kirouac, 2011; Van der Werf et al., 2002), as well as information regarding on-going motivated behavior via their inputs from basal ganglia output structures (O'Donnell et al., 1997). MTN efferents project to many of the same limbic structures that they receive input from (Berendse & Groenewegen, 1990; Li & Kirouac, 2007; Pinto et al., 2003; Su & Bentivoglio, 1990; Vertes & Hoover, 2008). Thus, MTN are uniquely situated to integrate information regarding the sensory and motivational state of the animal to guide behavioral responses to motivationally salient stimuli in the environment, such as reinstatement of cocaine-seeking.

Despite being highly interconnected with other structures involved in the reinstatement of cocaine-seeking, much less is known about the role of MTN in addiction-related behaviors. Recently it has been argued that PVT should be included in the addiction circuit (James & Dayas, 2013; Martin-Fardon & Boutrel, 2012). For example, an acute injection of a psychostimulant or exposure to a cocaine-paired

environment increases neuronal activation in PVT, as measured by induction of the immediate early gene cFos (Brown et al., 1992; Deutch et al., 1998; Franklin & Druhan, 2000; Johnson et al., 2010). Furthermore, lesions or inactivation of PVT blunts development of locomotor sensitization and blocks expression of a conditioned place preference to cocaine (Browning et al., 2014; Young & Deutch, 1998), suggesting PVT regulates the development and expression of non-contingent addiction behaviors. Importantly, MTN are implicated in reinstatement of drug-seeking following self-administration. For example, cue-induced reinstatement of cocaine seeking increases cFos in PVT, IMD, and MD (James et al., 2011; Matzeu et al., 2015), lesions of PVT blunt context-induced reinstatement of ethanol seeking (Hamlin et al., 2009), and intra-PVT inactivation by TTX attenuates cocaine-primed reinstatement (James et al., 2010).

However, understanding the exact contribution of MTN to relapse behaviors is difficult because MTN are highly interconnected with several other structures in the mesocorticolimbic circuit that differentially regulate reinstatement of cocaine-seeking. Considerably less is known about how MTN efferents to NAc (MTN-NAc) regulate reinstatement of cocaine-seeking. Context-induced reinstatement of ethanol seeking increases neuronal activation in PVT-NAc (Hamlin et al., 2009). Neumann et al (2016) utilized a combinatorial viral approach to abolish neurotransmission in these same neurons and found that this reduced cocaine self-administration but did not alter incubation of cocaine craving (Neumann et al., 2016). These two studies support the idea that MTN-NAc may regulate distinct aspects of addiction, such as self-administration and reinstatement. The experiments outlined in this chapter will extend

current knowledge of the contribution of MTN and MTN-NAc in two rodent models of relapse, cue-induced and drug-primed reinstatement of cocaine-seeking.

To investigate the role of MTN and MTN-NAc in reinstatement of cocaine-seeking, we used viral-mediated gene transfer techniques to express $G_{i/o}$ -DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) in these neurons. DREADDs are only activated by the otherwise inert ligand, clozapine-N-oxide (CNO), which allows for a specific and temporary increase in $G_{i/o}$ -signaling cascades in neurons expressing $G_{i/o}$ -DREADDs (Rogan & Roth, 2011; H. Zhu & Roth, 2014). Activation of $G_{i/o}$ -DREADDs by CNO decreases neuronal activity via a reduction in intracellular cAMP levels and activation of G-protein-coupled inwardly rectifying potassium channels (GIRK), which leads to hyperpolarization of the neuron and attenuation of firing (Armbruster et al., 2007; Ferguson et al., 2010; Kerstetter et al., 2015; Zhu & Roth, 2014). First, we expressed $G_{i/o}$ -coupled DREADD (hM4Di) in MTN neurons in rats to assess how transiently inhibiting neuronal activity via $G_{i/o}$ -coupled signaling cascades affects cue-induced and cocaine-primed reinstatement of drug-seeking. Second, we expressed hM4Di in MTN-NAc. We bilaterally injected a retrogradely transported, canine adenovirus (cAV) expressing cre-recombinase into the NAc and cre-dependent adenoassociated virus (AAV5) expressing hM4Di into MTN. This allowed us to reduce neuronal activity specifically in MTN-NAc during cue-induced and cocaine-primed reinstatement. We hypothesized that these manipulations would attenuate both cue-induced and cocaine-primed reinstatement of drug-seeking.

3.3 Materials and Methods

Animal use. All experiments were approved by the Seattle Children's Research Institute Institutional Animal Use and Care Committee and adhered to National Institutes of Health guidelines. Male Sprague Dawley rats (n=60, obtained from Envigo) weighing 250-274g upon arrival acclimatized to the environment for at least 3 days prior to any experimental manipulation. All rats were pair housed for the duration of the experiment. The housing environment was maintained on a 12h light/dark cycle and contained temperature and humidity control. Food and water was available *ad libitum* until 3 days prior to the start of behavior testing. Rats were then food restricted for the duration of the experiment, and rats were fed 16-20 g of rat chow once per day. Twenty-two rats were excluded from analysis because of catheter failures (n=9), inability to learn self-administration (n=3), resistance to extinction (n=3), and virus localization (n=7).

Drugs. Clozapine-N-oxide (CNO) was obtained from the National Institutes of Health. CNO was administered into the intraperitoneal cavity (*ip*) in a volume of 1 mL/kg at doses of 5 mg/kg or 10 mg/kg. CNO was dissolved in 100% DMSO, then diluted in sterile water for a final concentration of 6% DMSO for 5 mg/kg CNO or 12% DMSO for 10 mg/kg CNO doses. Vehicle injections consisted of either 6% or 12% DMSO mixed in sterile water; the concentration of DMSO used in the vehicle corresponded to the maximum dose of CNO used in an experiment. Cocaine HCl (obtained from National Institute of Drug Abuse) was dissolved in sterile 0.9% saline. Gentamycin (1 mg/kg IV), sodium brevitol (10 mg/mL IV), Baytril (20-25 mg/kg SC), meloxicam (0.2 mg/kg SC), beuthanasia, and isoflurane (2-4%, inhaled) were dissolved in 0.9% sterile saline as appropriate (all obtained from Patterson Veterinary).

Viral vectors. Non-cre dependent (AAV5-hysn-hM4Di-mCherry) and cre-dependent (AAV5-hysn-DIO-hM4Di-mCherry) hM4Di-DREADD viral vectors were developed by Brian Roth and packaged in adenoassociated virus (AAV, serotype 5) at the University of North Carolina viral vector core with an approximate titer of 1×10^9 viral genomes per μL . Canine adenovirus expressing cre-recombinase (CAV-cre) had a titer of approximately 2.5×10^9 viral genomes per μL was prepared as previously described (Kremer et al., 2000) and obtained from John F. Neumaier.

Surgical techniques. During all surgical procedures described, rats were anesthetized with isoflurane and received meloxicam injection prior to surgery for analgesia. Using standard stereotaxic procedures, 33-gauge needles attached to gas-tight syringes (Hamilton Company) were placed above the region of interest. The following stereotaxic coordinates relative to Bregma (in mm) were used for virus injections (presented as brain region, anteriorposterior, mediolateral, dorsalventral, injection volume in μL): MTN, -2.5, ± 0.5 , -6, 1; NAc, +1.8, ± 1.4 , -7.5, 0.5. All viruses were bilaterally infused in the region of interest over 1 min, and needles were left in place for an additional 5 minutes to allow for diffusion away from the injection site and through the injection track. For the non-specific approach, AAV5-hysn-hM4Di-mCherry was injected into MTN of all rats. For projection-specific experiments, AAV5-hysn-DIO-hM4Di-mCherry was injected into MTN and CAV-cre was injected into the NAc. All rats received hM4Di injections into MTN to allow for a within-subject experimental design. Immunohistochemistry against the mCherry tag on the hM4Di was performed to verify virus expression.

Rats had at least 3 days of post-operative recovery and monitoring following stereotaxic infusions. Rats were then anesthetized with isoflurane and implanted with jugular catheters. Briefly, chronic indwelling catheters were inserted into the right jugular vein and attached to a back-mounted port. Catheters were flushed daily with gentamycin, and rats received prophylactic injections of Baytril for at least 5 days post-operation. Catheter patency was assessed the day prior to the first session and after the last session of self-administration by injecting sodium breivital *iv*. To prevent damage to catheters from pair housing, catheters were capped with stainless steel caps.

Intermittent access to cocaine self-administration. All behavior testing was performed in a standard drug self-administration chamber with retractable levers and stimulus lights from MedAssociates Inc. Levers were placed on the wall opposite the house light and stimulus lights were placed above each lever. A syringe pump (located outside the box) delivered cocaine via tubing attached to the back port on the catheter. All tubing was attached to a suspended swivel, which allowed for rats to move freely within the chambers.

Rats first learned to lever press for cocaine. Two levers extended into the chamber. Depression of the active lever yielded a single cocaine infusion (0.4 mg/kg in 50 nL delivered over 2.8s; FR1) and illumination of the stimulus light above the lever (4 sec) whereas pressing the inactive lever had no consequence (i.e., no cocaine and no light). Additional presses on the active lever during the cue light presentation were recorded but did not yield cocaine (ie. 4 second time-out). Active lever location was counterbalanced across all rats. Session continued for a maximum of 2 hours or until

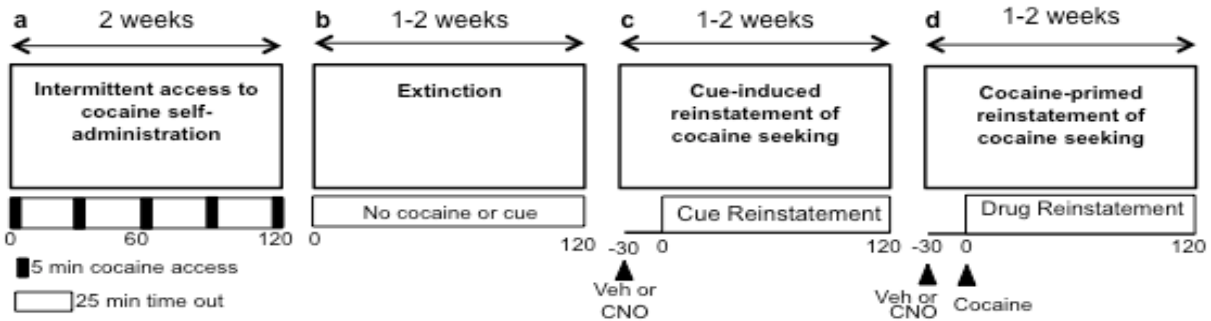


Figure 3.1. Experimental timeline and details. (a) Following training to lever press for cocaine, rats complete 14 sessions of intermittent access to cocaine self-administration (b) Rats undergo at least 7 days (or until active lever presses <25 for 2 consecutive days) of extinction training (c) Rats received counterbalanced *i.p.* injections of vehicle (5% DMSO) or CNO (5 or 10 mg/kg) 30 min prior to cue-induced reinstatement of cocaine-seeking testing sessions. Reinstatement tests are followed by at least 2 days of extinction training (or until extinction criterion are met) (d) Rats received a priming injection of cocaine (10 mg/kg) 30 min after an injection of vehicle or CNO. Test sessions are separated by extinction training. Veh=vehicle; CNO=clozapine-N-oxide

rats received 10 cocaine infusions. Rats underwent FR1 training for at least 5 sessions or until they met criterion (10 presses in less than 2 h).

Following completion of FR1 training, rats underwent 14 sessions of intermittent access to cocaine (Figure 3.1a). A single session consisted of 5 minutes access to cocaine followed by a 25-minute time-out period. This cycle repeated for a total of 125 min (five cocaine access periods and 4 time-outs). During the 5-minute access period, both levers were inserted into the chamber and a single press on the active lever yielded a single infusion of cocaine and presentation of the cue light as described above. Depression of the inactive lever resulted in no programmed consequences. A 4-second time-out period corresponded to the duration of cue-light presentation. After 5 minutes of access to cocaine, the levers retracted and the house light turned on for the duration of the 25-minute time-out period. An intermittent access cocaine self administration paradigm was used as it models human cocaine consumption and leads

to an enhanced motivation for drug-taking relative to standard continuous access paradigms (Zimmer et al., 2012b).

Extinction. Rats underwent extinction training at the conclusion of intermittent access cocaine self-administration (Figure 3.1b). Similar to FR1 training, 2 levers extended into the chamber for 2 h. However, presses on the active lever did not deliver cocaine nor activate the cue light. Extinction lasted for at least 7 days, or until rats pressed the active lever less than 25 times for 2 consecutive days.

Cue-induced reinstatement of cocaine-seeking. After lever pressing was extinguished, rats underwent 2 or 3 2-hour sessions (depending on the experiment) of cue-induced reinstatement of drug-seeking (Figure 3.1c). First, rats receive injections of vehicle or CNO 30 minutes prior to the start of the behavior session. Order of drug pre-treatment was counterbalanced across all rats. Rats were placed into the chambers, and upon extension of levers into the chambers, the cue light stimulus above the active lever came on for 10 s. Subsequent presses on the active lever resulted in a 4 s presentation of the cue light stimulus. Additional extinction training occurred between each cue-induced reinstatement session. Rats underwent extinction until criterion is met (2 consecutive days of less than 25 lever presses) in between each cue-induced reinstatement session.

Cocaine-primed reinstatement of cocaine-seeking. Rats received vehicle and CNO injections 30 min prior to the cocaine injection; drug pre-treatment was counterbalanced among all rats (Figure 3.1d). Then rats received a single cocaine injection (10 mg/kg cocaine *ip*), were transferred to the chambers, and underwent a

session identical to the extinction session described above. Between the two cocaine-prime sessions, rats underwent extinction training until extinction criteria are met.

Virus localization. Rats were anesthetized with Beuthanasia-D and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight, and sliced into 60 μm sections on a vibrating microtome. Floating sections were washed in 0.5% Triton-X/PBS solution for 10 minutes and blocked in 0.25% Triton-X/5% normal goat serum/PBS solution for 2 hours at room temperature. Sections incubated in 0.25% Triton-X/2.5% normal goat serum/primary antibody against mCherry (1:400, rabbit host, Clontech) solution over night at room temperature. Sections were rinsed in PBS, then incubated in a PBS/2.5% normal goat serum/goat anti-rabbit Alexa568 conjugated secondary antibody (1:400, Invitrogen) solution for 2 h at room temperature. Sections were rinsed in PBS, mounted onto slides, and cover slipped with Vectashield containing DAPI mounting medium (Vector Labs). Z-stacks were captured using a Zeiss LSM 710 Confocal microscope, and images were processed using ImageJ software (National Institutes of Health).

Data Analysis. All statistical analyses were determined prior to conducting experiments. GraphPad Prism 6 was used for statistical analyses. The effects of housing condition and session on earned infusions and active and inactive lever presses during self-administration and extinction were analyzed by mixed two-way analysis of variance (ANOVA) for housing condition (between-subjects variable) x session (within-subjects variable). Repeated measures phase x lever ANOVA tested the effects of cue or cocaine exposure on lever pressing in pair-housed, single-housed rats, and rats expressing hM4Di in MTN or MTN-NAc. The effect of housing condition on

lever pressing during extinction, cue-induced, or drug-primed reinstatement were analyzed by two-way mixed ANOVA for housing condition (between-subjects variable) x lever (within-subjects variable). Repeated measures treatment x lever ANOVA tested the effects of systemic CNO on lever pressing in rats expressing hM4Di in MTN and MTN-NAc neurons. All ANOVA analysis were followed by Dunnett or Sidak's multiple comparison's tests as appropriate. Average days to extinction are presented as mean±SEM (standard error of the mean).

3.4 Results

Characterization of relapse behaviors following intermittent access to cocaine self-administration in pair-housed rats. Since very few drug self-administration utilize pair-housed animals, we initially sought to characterize the self-administration, extinction, and reinstatement behavior of both single-housed and pair-housed rats undergoing intermittent access cocaine self-administration (IntA). Following training to lever press for cocaine, rats underwent 2 weeks of IntA (Figure 3.1a). We found no significant differences in the number of infusions (Figure 3.2a, no significant housing condition x session interaction, $F_{(13,507)}=0.50$, $P=0.92$; no main effect of housing, $F_{(1,39)}=2.62$, $P=0.11$) nor presses on the active (Figure 3.2b, no significant housing condition x session interaction, $F_{(13,507)}=0.32$, $P=0.99$; no main effect of housing, $F_{(1,39)}=2.64$, $P=0.10$) and inactive (Figure 3.2b, no significant housing x session interaction, $F_{(13,507)}=0.90$, $P=0.56$; no main effect of housing, $F_{(1,39)}=0.47$, $P=0.50$) levers between single- and pair-housed rats. Interestingly, we found that both single- and pair-housed rats escalate cocaine intake over the course of 2 weeks of IntA (Figure 3.2a,

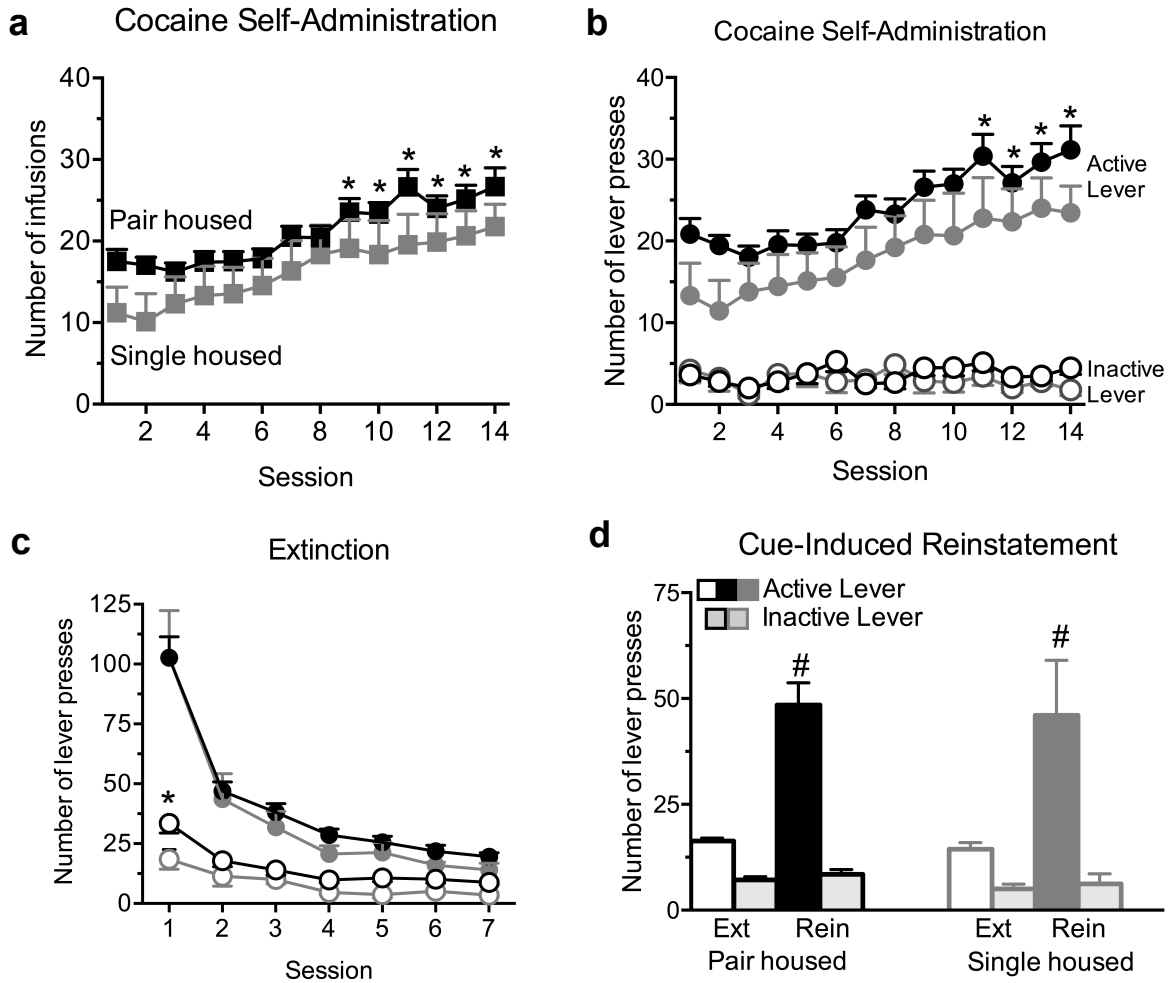


Figure 3.2 Intermittent access to cocaine self-administration leads to escalation of cocaine intake. (a) Rats escalate their cocaine intake over the two weeks of intermittent access to cocaine self-administration (IntA; * $p < 0.05$ compared to session 1 for both single- and pair-housed rats). There are no significant differences in the number of infusions earned between pair-housed ($n = 32$) and single-housed ($n = 9$) rats. (b) The number of active lever presses increase over the two weeks of IntA, and pair-housed rats have a similar number of active lever presses as single-housed rats (* $p < 0.05$ compared to session 1 for both single- and pair-housed rats). Inactive lever presses do not change over time nor does housing condition affect responses on the inactive lever. (c) Following IntA, rats extinguish responding on the active lever over the course of at least 7 days. There are no significant differences inactive lever pressing between single- and pair-housed rats. Pair-housed rats press significantly more on the inactive lever than single-housed rats during the first session of extinction. Inset: A small subset of the rats did not complete extinction ($n = 3$). The extinction-resistant rats had higher levels of active lever presses during the first extinction session (* $p < 0.05$) (d) Both pair- and single-housed rats show robust reinstatement of active lever pressing for presentation of a light cue associated with cocaine intake (# $p < 0.05$ compared to extinction).

main effect of session, $F_{(13,507)} = 14.91$, $P < 0.0001$). Both single- and pair-housed rats had

a higher number of cocaine infusions on sessions 9 through 14 when compared to session 1 (single and pair: $P < 0.05$ for each comparison). In line with an increase in the number of infusions across the 2 weeks of self-administration, we observed an escalation in the number of active lever presses (Figure 3.2b, main effect of session, $F_{(13,507)} = 11.89$, $P < 0.0001$) in both single- and pair-housed rats from sessions 11 through 14 (single and pair: $P < 0.05$ for each session compared to session 1). Pressing on the inactive lever did not change over time (no main effect of session $F_{(13,507)} = 0.85$, $P = 0.61$). Taken together, these data demonstrate that housing condition does not alter IntA. In addition, IntA leads to an escalation of cocaine intake, suggesting that IntA may lead to behaviors that increase vulnerability to compulsive cocaine use.

After completing 2 weeks of IntA, rats underwent extinction, where presses on the active lever have no programmed consequences (ie. no cocaine infusion or cue light). Rats were trained for at least 7 days with a criterion of less than 25 presses for 2 consecutive days (Figure 3.1b). Rats took on average 7.85 ± 0.28 days to meet extinction criterion; however data was analyzed for the first 7 days only since all rats completed at least 7 days of extinction training. It is interesting to note that a subset of all rats completing IntA (4/33 rats, or ~12%) were resistant to extinction (ie. did not meet extinction criteria within 16 sessions of extinction).

Housing condition had no overall effect on the number of active lever presses during extinction (Figure 3.2c; no housing x session interaction, $F_{(6, 234)} = 0.11$, $P = 0.99$; no main effect of housing, $F_{(1,39)} = 0.58$, $P = 0.45$). In addition, both single- and pair-housed rats exhibited a decrease in responses on the active lever (main effect of session, $F_{(6,234)} = 63.19$, $p < 0.0001$; single and pair: post hoc $P < 0.05$ for each session

compared to session 1). Housing condition altered inactive lever presses (no significant housing x session interaction, $F_{(6,234)}=1.21$, $P=0.30$; main effect of housing, $F_{(1,39)}=4.94$, $P=0.03$, and session $F_{(6,234)}=17.50$, $P<0.0001$). There was an effect on session 1, where inactive presses in pair-housed rats were higher than those made by single-housed rats ($t_{(273)}=3.42$, $P<0.05$). Despite this initial enhancement in inactive presses, pair-housed rats did not differ from single-housed rats in responding on subsequent days ($P<0.05$ for sessions 2-7).

Next we assessed whether housing condition altered cue-induced reinstatement of cocaine-seeking. Rats were placed into the behavior chambers, and the cue light previously paired with cocaine intake was presented to the rat. Subsequent presses on the active lever yielded presentation of the cue light (Figure 3.1c). This procedure models Pavlovian learning processes mediating relapse to cues associated with cocaine intake. Both pair- and single-housed rats reinstated responding on the active lever, as evidenced by higher pressing on the active lever during reinstatement than extinction (Figure 3.2d, Pair-housed: significant lever x phase interaction, $F_{(1,31)}=33.8$, $P<0.0001$; main effect of phase, $F_{(1,31)}=37.14$, $P<0.0001$ and main effect of lever, $F_{(1,31)}=105.2$, $P<0.0001$. Single housed: main effect of phase, $F_{(1,4)}=8.23$, $P=0.05$ and main effect of lever, $F_{(1,4)}=9.91$, $P=0.03$, no significant phase x lever interaction, $F_{(1,4)}=4.74$, $P=0.10$). Importantly, housing condition did not alter lever pressing during cue-induced reinstatement (Figure 3.2d, no significant housing x lever interaction, $F_{(1,35)}=0.0003$, $P=0.99$; no main effect of housing, $F_{(1,35)}=0.09$, $P=0.76$; main effect of lever, $F_{(1,35)}=33.40$, $P<0.0001$). Taken together, these data suggest that pair-housed rats show

similar patterns of IntA, extinction, and cue-induced reinstatement as single-housed rats. Therefore, rats were pair-housed for all subsequent experiments.

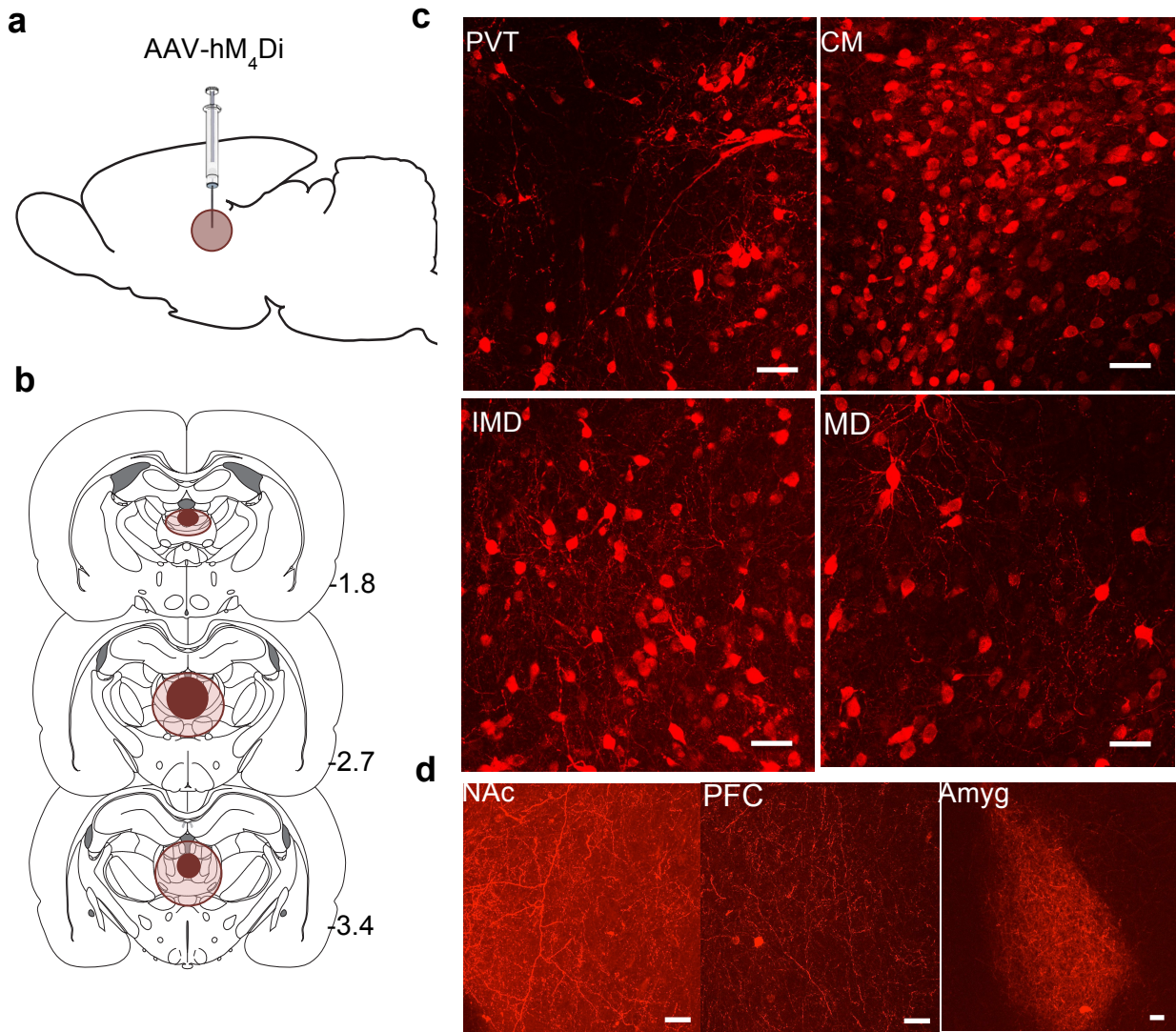


Figure 3.3. hM4Di expression in midline and intralaminar nuclei and its efferents. (a) An adeno-associated virus expressing Gi/o-coupled hM4Di DREADD under control of human synapsin I promoter was injected into midline and intralaminar nuclei. (b) Modified coronal sections from Paxinos and Watson rat brain atlas (2012) demonstrating maximal (light red shading) and minimal (dark red shading) expression of hM4Di in midline and intralaminar nuclei. (c) hM4Di expression in paraventricular (PVT), centromedian (CM), interomedian (IMD), and mediodorsal (MD) nuclei of the thalamus (d) hM4Di expression in axons or terminals in nucleus accumbens (NAc), prefrontal cortex (PFC), and amygdala (Amyg). Scale bars=50 μ m

Transgene expression occurs within midline thalamic nuclei and MTN efferents.

In order to determine the role of MTN in cue-induced and cocaine-primed reinstatement of cocaine seeking, we injected hM4Di-DREADD into several MTN (Figure 3.1c,d and Figure 3.2a). We achieved expression in primarily PVT, IMD, CM, and MD (Figure 3.3b,c). Additionally, since transgene expression can be detected within axons and terminals of infected neurons following a six-week incubation period and our behavior studies took at least 8 weeks to complete, we assessed hM4Di expression in several nuclei throughout the corticomesolimbic circuit associated with addiction. We saw high levels of fluorescence within the NAc, PFC, and AMYG (Figure 3.3d), suggesting MTN neurons project widely throughout the addiction circuit.

Reducing activity of midline thalamic nuclei attenuates relapse behaviors.

Following IntA and extinction training, we assessed the role of MTN during cue-induced reinstatement of drug-seeking. Rats received counterbalanced injections of vehicle, 5 mg/kg CNO, and 10 mg/kg CNO (*ip*) prior to a session testing cue-induced reinstatement of cocaine seeking, in which the rat is exposed to a cue previously paired with cocaine infusions and subsequent presses on the active lever yield presentation of this same cocaine-paired cue (Figure 3.1c). As expected, rats pressed the active lever significantly more during the cue-induced reinstatement session following a vehicle injection compared to pressing during extinction, indicating that exposure to cues associated with drug-taking led to cocaine-seeking behavior (Figure 3.4a; significant phase x lever interaction, $F_{(1,8)}=24.91$, $P=0.0011$; significant main effect of phase, $F_{(1,8)}=34.73$, $P=0.0004$ and lever, $F_{(1,8)}=110.5$, $P<0.0001$). Reducing activity of MTN

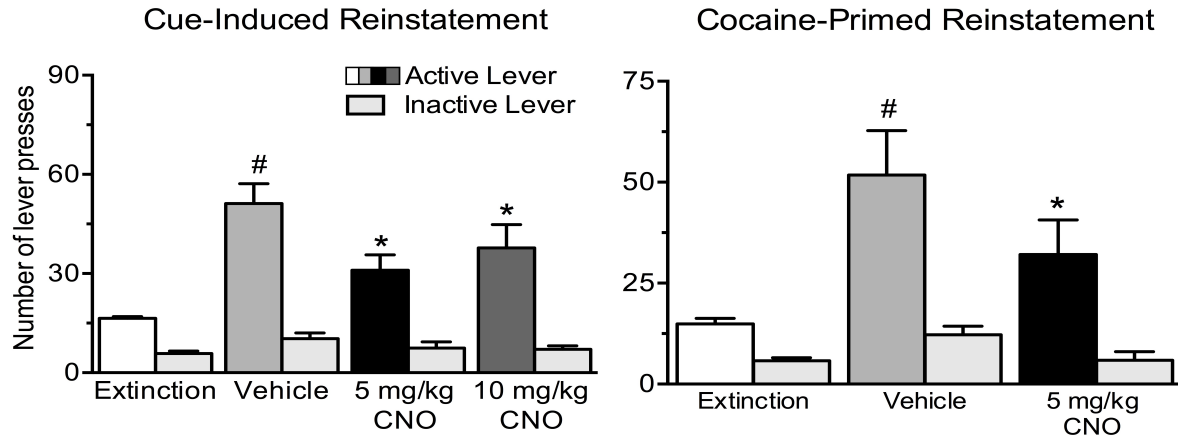


Figure 3.4 Midline and intralaminar nuclei (MTN) regulate relapse. (a) Reinvigoration of active lever pressing occurs when rats lever press for cues associated with cocaine-intake following vehicle injections ($n=9$, # $p<0.05$ compared to extinction). Dampening activity of MTN by pretreatment with either 5 or 10 mg/CNO attenuated active lever pressing during cue-induced reinstatement (* $p<0.05$ compared to vehicle lever presses) (b) Rats show robust responding on the active lever following a priming injection of 10 mg/kg cocaine (# $p<0.05$ compared to extinction). Activation of hM4Di by CNO attenuated active lever responses following a cocaine injection.

following injections of either 5 mg/kg or 10 mg/kg of CNO attenuated cue-induced reinstatement of cocaine-seeking (Figure 3.4a; significant main effect of treatment, $F_{(2,16)}=4.11$, $P=0.04$ and lever, $F_{(1,8)}=103.4$, $P<0.0001$; no significant treatment x lever interaction, $F_{(2,16)}=2.70$, $P=0.097$). This effect was due to a reduction in lever pressing on the active lever (5 mg/kg CNO: $t_{(16)}=3.82$, $P=0.003$; 10 mg/kg CNO: $t_{(16)}=2.54$, $P=0.04$) and not the inactive lever (5 mg/kg CNO: $t_{(16)}=0.55$, $p=0.83$; 10 mg/kg CNO: $t_{(16)}=0.61$, $P=0.80$), suggesting the manipulation did not have any non-selective effects on the ability of the rat to discriminate between the active and inactive lever.

We then tested whether MTN regulate another type of relapse behavior, drug-primed reinstatement. Rats received counterbalanced injections (*ip*) of vehicle or 5 mg/kg CNO thirty minutes prior to a cocaine injection (10 mg/kg, *ip*). Rats were then placed into the operant chambers, and lever presses on the active and inactive lever

were recorded (Figure 3.1d). When rats received vehicle injections prior to the cocaine prime, they show significantly higher levels of lever pressing on the active lever compared to extinction (Figure 3.4b; significant phase x lever interaction, $F_{(1,8)}=10.47$, $P=0.01$; significant main effect of phase, $F_{(1,8)}=14.7$, $P=0.005$; significant main effect of lever, $F_{(1,8)}=19.48$, $P=0.002$). When activity of MTN was reduced with a systemic injection of 5 mg/kg CNO (*ip*), drug-seeking was attenuated following a priming injection of cocaine (significant main effect of treatment, $F_{(1,8)}=10.14$, $P=0.01$; significant main effect of lever, $F_{(1,8)}=18.8$, $P=0.003$; no significant lever x treatment interaction, $F_{(1,8)}=2.85$, $P=0.13$). Reducing activity of midline thalamic nuclei selectively reduced pressing on the active lever ($t_{(8)}=3.52$, $P<0.05$) and not the inactive lever ($t_{(8)}=1.13$, $P>0.05$).

Transgene expression in midline thalamic nuclei neurons projecting to nucleus accumbens. We utilized a combinatorial viral mediated gene transfer approach in order to express hM4Di-DREADD in MTN neurons that project to NAc (MTN-NAc). A retrogradely transported canine adenovirus expressing cre-recombinase was injected bilaterally into NAc. An adeno-associated virus (serotype 5) expressing a cre-dependent hM4Di construct was injected into MTN. This approach allows us to selectively and transiently reduce neuronal activity in only MTN-NAc (Figure 3.5a). Using the combinatorial approach, we were able to achieve robust expression within the PVT, IMD, and CM nuclei (Figure 3.5b,c). We had little to no expression within MD (data not shown).

We examined sections from the NAc, PFC, and AMYG for transgene expression in axons in these regions. As expected, we saw robust expression in axons and/or

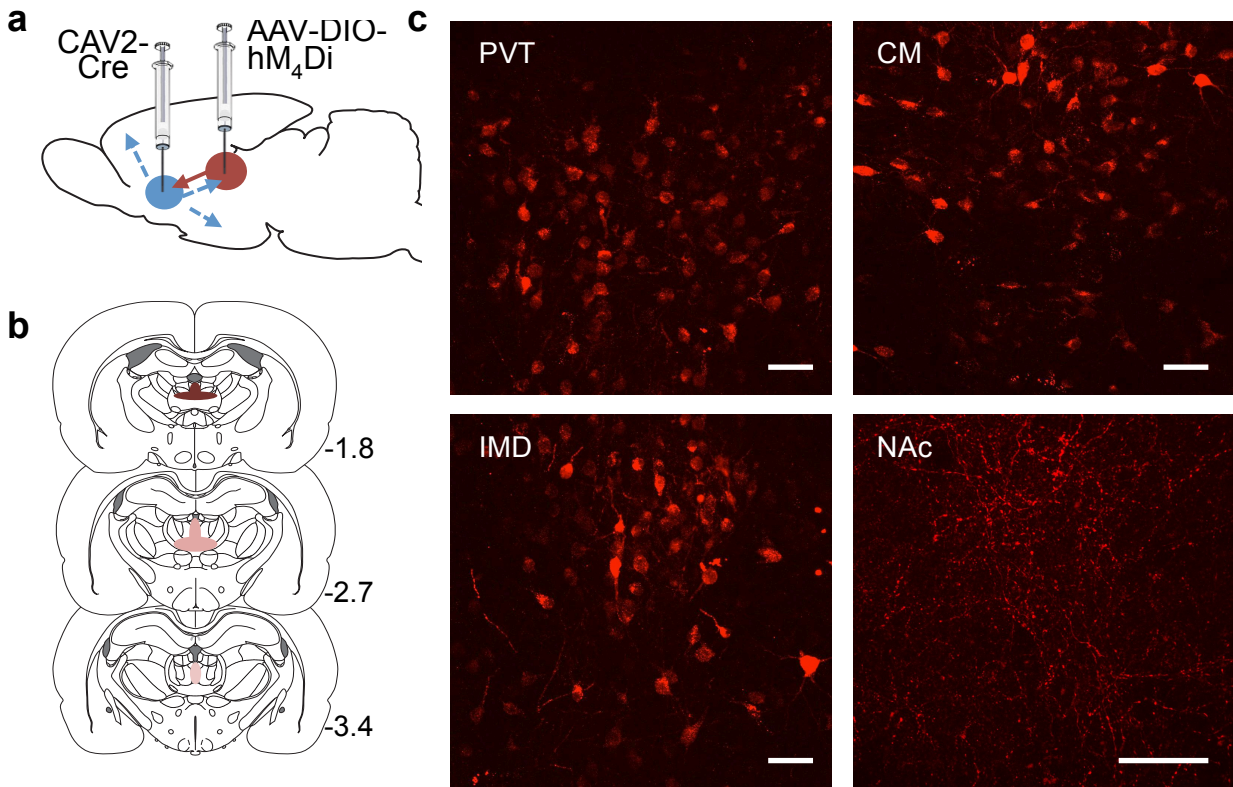


Figure 3.5 hM₄Di expression in thalamostriatal neurons. (a) Retrogradely transported canine adenovirus expressing cre-recombinase (cAV-cre) was injected into bilateral nucleus accumbens (NAc) and an adeno-associated virus expressing a cre-dependent hM₄Di DREADD was injected into midline and intralaminar thalamic nuclei (MITN). This allowed for expression of hM₄Di in only MITN neurons projecting to NAc. (b) hM₄Di expression was throughout MITN. Dark circles represent minimal expression for inclusion in data set and light shading represents maximal expression. (c) Immunohistochemical analysis of tissue demonstrating average hM₄Di expression expression in paraventricular (PVT), centromedian (CM), and interomediodorsal (IMD) nuclei. hM₄Di expression was detected in axons and/or terminals within the NAc. Scale bars=50 μ m

terminals in the NAc (Figure 3.5c). Interestingly, we also observed sparse, inconsistent labeling in axons with the PFC (data not shown), and no fluorescence was observed in the AMYG (data not shown), suggesting that MTN neurons projecting to the NAc do not send axon collaterals to these other brain regions.

Activation of anterior midline thalamic nuclei enhances cue-induced and abolishes cocaine-primed reinstatement. Previous research shows that glutamatergic signaling within the NAc regulates both cue-induced and drug-primed reinstatement

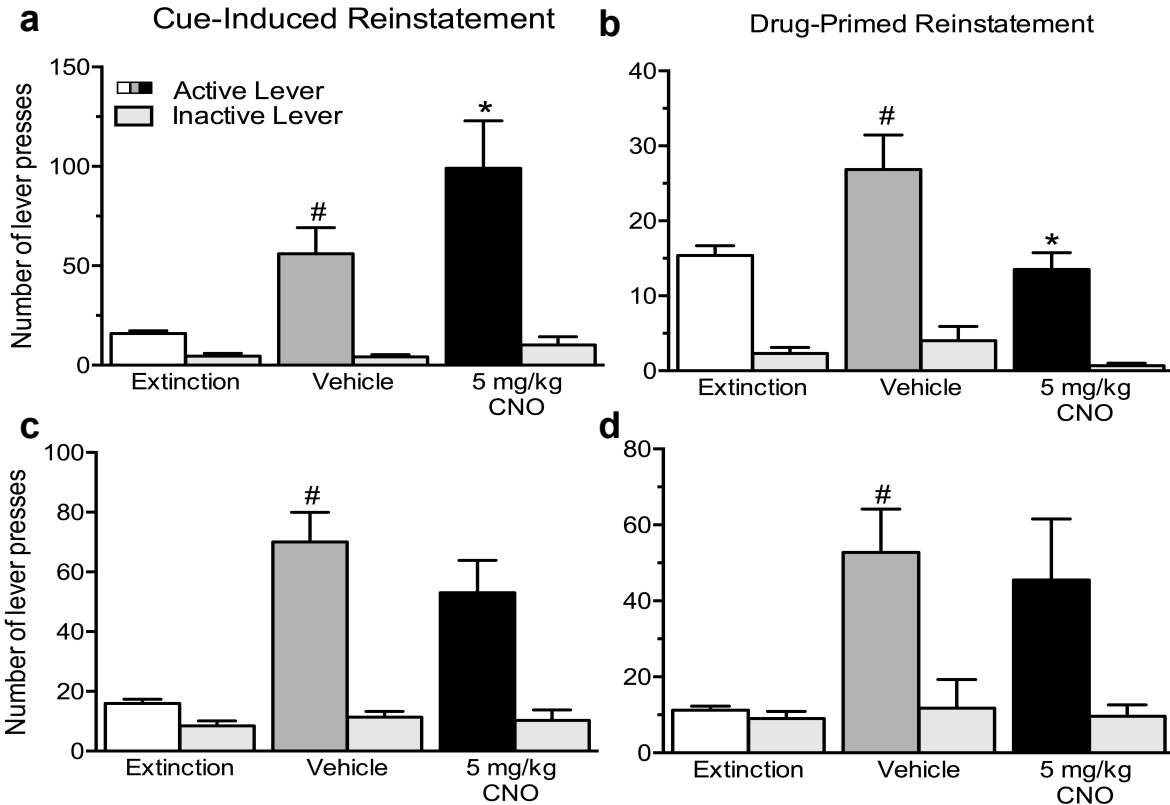


Figure 3.6 Activation of anterior midline thalamic nuclei enhances cue-induced and abolishes cocaine-primed reinstatement. (a) When pretreated with vehicle, rats reinstated responding on the active lever during cue-induced reinstatement test ($n=6$, # $p<0.05$ compared to extinction active lever pressing). Activation of hM4Di in anterior thalamostriatal neurons by administering CNO prior to cue-induced reinstatement enhanced presses on the active lever (* $p<0.05$ compared to vehicle). (b) Rats show an increase in active lever pressing when injected with vehicle prior to a 10 mg/kg injection of cocaine ($n=6$, # $p<0.05$ compared to extinction active lever pressing). Activating $G_{i/o}$ -signaling cascades prior to the cocaine-priming completely abolished responding on the active lever (* $p<0.05$ compared to vehicle). (c) Following vehicle injections, rats reinstate during cue-induced reinstatement ($n=8$, # $p<0.05$ compared to extinction). Dampening activity of posterior thalamostriatal neurons had no effect on cue-induced reinstatement of cocaine-seeking. (d) While rats reinstate responding on the active lever in response to a priming injection of cocaine following vehicle injections (# $p<0.05$ compared to extinction), attenuating activity of posterior thalamostriatal neurons did not alter drug-primed reinstatement.

(McFarland et al., 2003; Park et al., 2002; review see Yager et al., 2015). Because MTN are a dense source of NAc glutamate (Berke & Hyman, 2000; Lüscher & Malenka, 2011; Nestler, 2001; Russo et al., 2010; Schmidt & Pierce, 2010; Steketee & Kalivas,

2011), and we observed strong fluorescence of axons within the NAc, we sought to explore whether MTN-NAc were responsible for the attenuation in cocaine-seeking we observed with the non-selective approach. Therefore, we selectively attenuated MTN-NAc activity with hM4Di during both cue-induced and drug-primed reinstatement of cocaine-seeking. Rats received counterbalanced injections of vehicle or 5 mg/kg CNO (*ip*) prior to cue-induced and cocaine-primed reinstatement sessions (Figure 3.1c,d). We split rats into two groups based on expression in anterior or posterior MTN-NAc, based on previous work by (Li & Kirouac, 2007): anterior group had maximal expression from Breg -1.8 to -2.5 (antMTN-NAc) and those in posterior subdivision had maximal hM4Di expression from Breg -2.3 to 3.3 (postMTN-NAc). hM4Di expression did not extend into the most posterior portion of MTN (Breg -3.5 to -4.0) in MTN-NAc rats. Unexpectedly, although these rats exhibited reinstatement to cues associated with cocaine intake following vehicle injections (Figure 3.6a; significant phase x lever interaction, $F_{(1,5)}=10.60$, $P=0.02$; significant main effect of phase, $F_{(1,5)}=9.43$, $P=0.03$; significant main effect of lever, $F_{(1,5)}=26.08$, $P=0.004$), activation of hM4Di with a systemic CNO injection (5 mg/kg, *ip*) significantly enhanced active lever pressing for cocaine-associated cues compared to vehicle treatment (significant treatment x lever interaction, $F_{(1,5)}=7.56$, $P=0.04$; significant main effect of lever, $F_{(1,5)}=21.16$, $P=0.006$; trend towards significant main effect of treatment, $F_{(1,5)}=5.53$, $P=0.06$). Nonetheless, attenuating activity of antMTN-NAc abolished cocaine-primed reinstatement (Figure 3.6b; significant main effect of treatment, $F_{(1,5)}=12.56$, $P=0.02$; main effect of lever, $F_{(1,5)}=35.42$, $P=0.002$; no significant treatment x lever interaction, $F_{(1,5)}=2.88$, $P=0.15$) whereas rats reinstated pressing on the active lever following a priming injection of cocaine (Figure

3.7b; significant main effect of phase, $F_{(1,5)}=8.98$, $P=0.03$; significant main effect of lever, $F_{(1,5)}=37.95$, $P=0.002$; no significant phase x lever interaction, $F_{(1,5)}=3.72$, $P=0.11$). In contrast, dampening activity of postMTN-NAc had no effect on cue-induced (Figure 3.6c; no significant treatment x lever interaction, $F_{(1,7)}=1.55$, $P=0.125$; no significant main effect of treatment, $F_{(1,7)}=0.95$, $P=0.37$; main effect of lever, $F_{(1,7)}=79.27$, $P<0.0001$) or cocaine-primed reinstatement (Figure 3.6d; no significant treatment x lever interaction, $F_{(1,7)}=0.15$, $P=0.71$; no significant main effect of treatment, $F_{(1,7)}=0.27$, $P=0.62$; main effect of lever, $F_{(1,7)}=8.62$, $P=0.02$).

3.5 Discussion

In this set of experiments, we utilized chemogenetic and viral mediated gene transfer techniques in order to more clearly define the role of MTN in rodent models of relapse. First, we found that pair-housing rats did not alter IntA, extinction, or cue-induced reinstatement of cocaine-seeking. Interestingly, rats undergoing IntA exhibit an escalation of cocaine intake and a subset of these rats are extinction-resistant (i.e. did not meet extinction criterion). Of note, these rats also have higher responding on the first day of extinction. Next, we found that increasing $G_{i/o}$ -signaling cascades in MTN via activation of hM4Di-DREADDs attenuated both cue-induced and cocaine-primed reinstatement of drug-seeking. Because MTN are a large source of striatal glutamate, we examined whether $G_{i/o}$ -signaling cascades within thalamostriatal neurons contributed to drug-seeking behavior. Surprisingly, we found MTN-NAc regulate reinstatement behaviors in a complex, location-specific manner. Namely, dampening activity of anterior MTN-NAc enhanced cue-induced reinstatement but reduced cocaine-

primed reinstatement. The same manipulation in posterior MTN-NAc had no effect on reinstatement. Taken together, these results highlight the contribution of MTN to relapse behavior.

We utilized a recently developed intermittent access to cocaine self-administration (IntA) paradigm for our studies, which allows for a binge-like pattern of drug intake and a “spiking” pattern in the estimated concentration of dopamine in the brain of rats self-administering cocaine (Zimmer et al., 2012b). We demonstrate for the first time that IntA leads to an escalation of cocaine intake, previously only observed following extended access to cocaine (Ahmed, 1998; Edwards & Koob, 2013; Lesscher & Vanderschuren, 2012). However an alternative explanation is the escalation observed here may simply reflect the learning of the IntA protocol. For example, the rats shift from 2 h continuous access in FR1 training to the 5 min access/25 min timeout cycle in session 1 of self-administration. This is unlikely the case, as IntA leads to an enhanced motivation for cocaine intake that is also observed in extended access paradigms and is a key criterion associated with addiction (Edwards & Koob, 2013; Lesscher & Vanderschuren, 2012; Paterson & Markou, 2003; Zimmer et al., 2012b). In addition, rats that escalate their cocaine intake exhibit enhanced drug-primed, cue-induced, and stress-induced reinstatement behavior, suggesting that escalation can be an early indicator of increased vulnerability to relapse (Deroche et al., 1999; Kippin et al., 2006; Mantsch et al., 2004; Vanderschuren, 2004). In fact, one study utilizing a modified IntA paradigm with shorter timeout periods (4-6 min) demonstrated that rats with a short inter-infusion interval (ie. high frequency intake) had higher levels of cocaine-primed reinstatement compared to rats that had a lower frequency of infusions, suggesting that

IntA may lead to an enhancement in reinstatement behaviors (Martin-Garcia et al., 2014). In fact, a small subset of rats undergoing IntA were resistant to extinction; these rats pressed more on the active lever during the first session of extinction compared to rats that successfully met extinction criteria. However, it remains to be determined whether drug-seeking and drug-craving (ie. incubation of drug craving) behaviors are enhanced in IntA compared to short or long access self-administration paradigms, which would further validate IntA as a model of compulsive addiction behaviors. Nonetheless, it is paramount that pre-clinical models of cocaine intake accurately reflect the pattern of drug intake in the addicted population, and these data suggest rats that undergo “binge-like” cocaine intake show behaviors such as enhanced motivation for drug-taking, escalation of drug intake, and resistance to extinction, key factors that precede or contribute to compulsive drug use.

One difference in our experimental design is that we chose to pair-house the rats for the duration of the self-administration experiment. Previous studies indicate that rats housed in isolation do not have higher drug intake during self-administration, but are more sensitive to the rewarding effects of cocaine at low doses compared to socially housed rats (Bardo et al., 2001; Bozarth et al., 1989; Howes et al., 2000; Schenk et al., 1988). Isolated rats exhibit resistance to extinction and enhanced cue-induced and drug-primed reinstatement compared to rats in enriched environments (Thiel et al., 2010; Thiel et al., 2009). Moreover, single housed rats have an increase in basal dopamine neurotransmission and enhanced dopamine release, blunted glutamate signaling, and an overactive stress axis in response to psychostimulants (Bowling et al., 1993; Melendez et al., 2004; Rahman & Bardo, 2008; Serra et al., 2009). These data

suggest that isolation housed rats are hypersensitive to the rewarding effects of cocaine, more prone to relapse, and have profound changes in neurotransmission linked with a propensity for addiction (Bardo et al., 2012; Neisewander et al., 2012). Therefore, we hypothesized that pair-housed rats may show reduced cocaine intake and relapse behaviors compared to single-housed rats. Quite surprisingly, we demonstrate no significant differences in the IntA self-administration, extinction, and cue-induced and drug-primed reinstatement of cocaine-seeking behaviors in pair-housed rats compared to single-housed rats. However, this is in line with a single study showing no difference in extinction, cue-induced, or drug-primed reinstatement in isolated vs pair-housed rats (Thiel et al., 2009). Nonetheless, we advocate for group housing rodents undergoing IntA self-administration not only because it may avoid profound alterations in basal neurotransmission seen in isolated rats but more closely reflects the social nature of humans and rodents throughout their daily lives.

The main goal of this work was to explore the role of $G_{i/o}$ -signaling cascades in midline and intralaminar thalamic nuclei (MTN). We injected an adenovirus expressing $G_{i/o}$ -coupled DREADD (hM4Di) into MTN; specifically, all rats exhibited expression of hM4Di in paraventricular (PVT), mediodorsal (MD), interomediodorsal (IMD), and centromedian (CM) nuclei of the thalamus. Activation of hM4Di with CNO in MTN leads to a reduction in neuronal activation (Zhu et al., 2016). We found that reducing MTN activity via hM4Di attenuated both cue-induced and drug-primed reinstatement of cocaine seeking. This result is consistent with the idea that MTN contribute to relapse behavior. For example, markers of neuronal activation (ie. cFos) increase in MTN following exposure to visual, olfactory, or contextual cues paired with either cocaine or

alcohol self-administration (Dayas et al., 2008; Hamlin et al., 2009; James et al., 2011; Matzeu et al., 2015; Wedzony et al., 2003). Lesion or inactivation studies have demonstrated a reduction in context-induced and drug-primed reinstatement of cocaine and ethanol seeking (Hamlin et al., 2009; James et al., 2010). Furthermore, expression of conditioned place preference and context-dependent sensitization are disrupted following lesions of MTN (Browning et al., 2014; Young & Deutch, 1998). These regions have also been implicated in regulating responses to cues associated with highly palatable food rewards (Kelley et al., 2005; Schiltz et al., 2007). Temporary inactivation via hM4Di confirmed prior studies demonstrating the importance of MTN to cocaine-primed reinstatement, and extended the literature to define a role of these nuclei in cue-induced reinstatement of cocaine-seeking. Thus, it appears that MTN regulate cue-motivated behaviors such as relapse.

Although the non-selective DREADD approach demonstrates a general role of MTN in addiction-related behaviors, we cannot make any meaningful conclusions about which particular sets of MTN efferents are responsible for regulating relapse since all outputs would be equally affected by this manipulation. Indeed, we examined hM4Di expression in axon terminals throughout the cortico-basal ganglia circuit and consistent with prior tracing studies we demonstrate that MTN project to NAc, PFC, AMYG, and dorsal striatum (Berendse & Groenewegen, 1990; Li & Kirouac, 2007; Pinto et al., 2003; Su & Bentivoglio, 1990; Van der Werf et al., 2002; Vertes & Hoover, 2008). Even though our manipulation has better temporal specificity compared to other studies, it is likely that we affected MTN output to several downstream regions. In fact, using a similar approach in the PFC, we found that activation of hM4Di in those neurons not only

reduced neuronal activation in PFC but also in downstream medium spiny neurons (Kerstetter et al., 2015). Thus it is likely our manipulation in MTN may have altered activity in other downstream structures closely related to relapse.

Because glutamatergic signaling in NAc regulates relapse, and MTN neurons send a dense glutamatergic projection to NAc (Berendse & Groenewegen, 1990; Frassoni et al., 1997; Kalivas & Volkow, 2011; Li & Kirouac, 2007; Steketee & Kalivas, 2011), we tested the hypothesis that MTN-NAc contribute to both cue-induced and drug-primed reinstatement. We used a combinatorial viral approach to selectively express hM4Di in MTN-NAc neurons by injecting cAV-cre into bilateral NAc and an AAV expressing a cre-dependent hM4Di into MTN. This approach led to hM4Di expression primarily in PVT, CM, and IMD, with little to no expression in MD. This is consistent with studies showing PVT and IMD project primarily to medial NAc whereas PVT and CM project to lateral NAc following cholera toxin B injections (Berendse & Groenewegen, 1990). Thus, it's likely cAV-cre infected neurons throughout both NAc core and shell regions. In fact, we detected robust labeling of hM4Di in axons in the NAc using the combinatorial technique, verifying cAV-cre placement within NAc. Furthermore, there appears to be an anterior to posterior topography in the projection from MTN to NAc, such that anterior PVT projects to NAc shell, and IMD, CM, and central and posterior PVT project primarily to NAc core (Li & Kirouac, 2007). Therefore, we assessed the effects of dampening activity in anterior and posterior MTN-NAc on reinstatement behaviors.

Activation of hM4Di in antMTN-NAc by systemic CNO injection completely abolished reinstatement of cocaine-seeking following a priming injection of cocaine.

Thus, it is likely that activity in antMTN-NAc neurons are sufficient to drive thalamic-mediated aspects of cocaine-primed reinstatement behavior in rats. Because these antMTN-NAc neurons preferentially project to NAc shell, it's likely our effects were due to MTN projections to NAc shell (Li & Kirouac, 2007). In addition, MTN-NAc neurons not only converge onto dendritic spines on medium spiny neurons but also onto dopamine terminals, and electrical stimulation of MTN leads to glutamate-dependent increases in dopamine release in the NAc (Parsons et al., 2006; Pinto et al., 2003). Thus, dampening activity of glutamatergic MTN-NAc reduced both glutamatergic and dopaminergic influences onto medium spiny neurons that lead to a decrease in the output of medium spiny neurons and the abolishment of drug-primed reinstatement.

In direct contrast to the drug-prime reinstatement data, we found that attenuating activity of antMTN-NAc enhanced cue-induced reinstatement of cocaine-seeking, suggesting antMTN-NAc may regulate cue-induced reinstatement via a different mechanism. Not only do MTN neurons synapse directly onto medium spiny neurons, they are also the main source of glutamate onto cholinergic interneurons (AChI) in the striatum (Doig et al., 2014). In response to stimulation of thalamic inputs in a manner that mimics the firing pattern to salient environmental cues (such as cues associated with cocaine-intake), AChI fire in a burst, which is followed by a long (0.5 sec) pause (Ding et al., 2010; Doig et al., 2014). This leads to a dampening of corticostriatal inputs onto all medium spiny neurons during the burst and longer inhibition in the activity of striatopallidal neurons (Ding et al., 2010). This inhibition of striatopallidal neurons could shift the balance of striatal outputs to the striatonigral neurons, thus leading to an enhancement in cue-induced reinstatement of drug-seeking. Furthermore, a recent

study demonstrated that antagonism of nicotinic receptors in NAc during a Pavlovian instrumental transfer task enhances cue-induced reinstatement of reward seeking, suggesting the effects of our manipulation may have interfered with cholinergic actions on nicotinic receptors (Collins et al., 2016).

We found no effect of our manipulation in postMTN-NAc. Given that postMTN-NAc neurons project primarily to NAc core and glutamatergic signaling in the NAc core is highly implicated in relapse behaviors, this lack of effect is intriguing (Li & Kirouac, 2007; Steketee & Kalivas, 2011). Nonetheless, this finding further supports the idea that MTN-NAc regulate addiction in a complex manner and care should be taken to consider the exact location of the manipulation. For example, a recent study demonstrated that PVT neurons projecting to NAc regulate cocaine self-administration but not incubation of cocaine craving (Neumann et al., 2016). This group did achieve expression in anterior PVT, however expression corresponded to posterior MTN regions studied here, which may account for the lack of effect on cue-induced cocaine craving in this experiment. Furthermore, optogenetic stimulation of PVT neurons projecting to NAc was shown to be aversive and that changes in plasticity in these neurons contribute to opioid withdrawal (Zhu et al., 2016). It is important to note that these two studies were able to specifically isolate PVT neurons projecting to NAc, whereas studies described here also targeted IM and CM nuclei. Future studies will need to be conducted to isolate specifically IM and CM neurons projecting to NAc in order to better understand how MTN-NAc regulate relapse. Thus, the exact role of MTN-NAc in regulating addiction may be complex, but it is clear these neurons are important regulators of addiction.

In summary, this research demonstrates how MTN and MTN-NAc regulate relapse behaviors. Our results suggest that MTN may be a key node for regulating cue-induced and cocaine-primed reinstatement of cocaine-seeking. In particular, MTN neurons projecting to NAc seem to be especially critical for reinstatement. Given that relapse is triggered by drug-associated stimuli and the fact that relapse rates remain high in addicts, this work has important clinical implications in that it suggests MTN may be a promising therapeutic target.

Chapter 4

Conclusions and Future Directions

4.1. Summary of key findings

In this thesis, I explored the role of cortical and thalamic inputs into the nucleus accumbens (NAc) during addiction-related behaviors. While the cortex and thalamus have been implicated in regulating addiction behaviors, many of these previous studies utilized manipulations that were lacking either temporal or spatial specificity. Consequently, this has resulted in ambiguity as to these two NAc glutamatergic afferents regulate addiction. To circumvent this issue, I utilized a recently developed combinatorial viral mediated gene transfer technique paired with chemogenetics-which allows for transient inactivation of a defined set of cells-to explore the role of corticostriatal and thalamostriatal neurons in regulation of psychomotor sensitization, drug-taking, and drug-seeking behaviors.

Chapter 2 focused on establishing the role of corticostriatal afferents in psychomotor sensitization, drug-taking, and drug-seeking. First, I show that dampening activity of corticostriatal neurons during repeated amphetamine treatment attenuated the development of psychomotor sensitization and blunted conditioned locomotor responses during a subsequent challenge session. Second, the same manipulation during a cocaine self-administration paradigm did not alter cocaine-taking. However, performing this manipulation during drug-taking led to a subsequent impairment of

extinction and enhanced responding during cocaine-primed reinstatement. Furthermore, reducing activity of corticostriatal neurons prior to a priming injection of cocaine blunted cocaine-primed reinstatement of drug-seeking. Taken together, these results revealed a novel role for corticostriatal neurons in regulating responses to drugs and drug-associated stimuli.

Chapter 3 was devoted to establishing a role of midline thalamic nuclei (MTN) and thalamostriatal neurons in the regulation of relapse behaviors. Despite being a dense source of glutamate into the NAc, direct behavioral evidence identifying a role of MTN in cocaine relapse behaviors is lacking. The third major finding of my work was that reducing activity of MTN attenuated both cue-induced and cocaine-primed reinstatement by reducing activity of MTN prior to reinstatement, which establishes a role of MTN in regulating responses to both external and internal cues associated with cocaine intake. Lastly, I demonstrated the complex nature of thalamostriatal neuronal regulation of relapse behavior. Specifically, I demonstrated that dampening activity of thalamostriatal neurons in the anterior portion of MTN nuclei abolished cocaine-primed reinstatement but enhanced cue-induced reinstatement. There was no effect of this manipulation in thalamostriatal neurons located in the posterior MTN. Taken together, these results suggest that the often overlooked MTN, and in particular anterior thalamostriatal projection neurons, should be included in the addiction circuit as they are an important modulator of relapse behaviors.

4.2. Impact of this work and future directions

Experimental approach. The experiments in this thesis used recently developed combinatorial viral approaches paired with chemogenetics in order to selectively modulate neuronal activity in specific sets of projection neurons in rodent models of relapse. Because these studies are among the first to utilize these techniques in rats, it is prudent to consider the advantages and disadvantages of this approach.

Specifically, I used the hM4Di-DREADD which is activated by the otherwise inert ligand CNO. This activates $G_{i/o}$ -signaling cascades that increases GIRK channel function to hyperpolarize neurons and blunt activity (Armbruster et al., 2007; Ferguson et al., 2010). There have been numerous examples using a variety of techniques (ie. slice electrophysiology, *in vivo* recordings, EEG, fMRI) in several different brain regions and cell types, and all have demonstrated hM4Di dampen neuronal activity (for review see Roth, 2016). While the kinetics and pharmacology of CNO activation of hM4Di receptors may vary across brain region and cell types, it is commonly understood that maximal effects are seen at least 20 minutes following systemic CNO injection and last for up to 90 minutes (Smith et al., 2016). Thus, chemogenetic tools are a minimally invasive (ie. requires only viral injections and systemic CNO injections) method for transiently reducing neuronal activation for a duration that is conducive to studying more complex rodent behaviors such as models of addiction (Ferguson & Neumaier, 2015; Roth, 2016; Smith et al., 2016). However, GPCR intracellular signaling cascades (such as $G_{i/o}$ cascade activated by hM4Di) are known to have complex pharmacology with a diverse range of effects that impact cellular function. Thus it is likely there are other downstream effects of hM4Di manipulation than just simply reducing neuronal activity,

although these effects have yet to be explored or determined (Bradley & Tobin, 2016; Roth, 2016). Despite ample evidence indicating hM4Di reduces neuronal activation, in chapter 2 I demonstrated that CNO injections prior to an acute injection of cocaine attenuated cocaine-induced increases in cFos in PFC. This confirmed that activation of hM4Di does indeed blunt neuronal activation in glutamatergic neurons, and the behavioral effects described throughout this thesis are likely attributable to decreased neuronal activity.

This work paired chemogenetics with a viral approach in order to achieve spatial specificity of the manipulation (ie. hM4Di expression in defined sets of NAc afferents). Adeno-associated viruses (AAV) exhibit stable, long-lasting expression, which gives ample time for transgenes to be trafficked along the entirety of a neuron, including axons, thus allowing for analysis of where infected neurons project throughout the brain. I demonstrate in chapter 2 that corticostriatal neurons project primarily to NAc but also send collaterals to basolateral AMYG. Furthermore, MTN neurons project to NAc, AMYG, and PFC (Chapter 3). This raised the possibility that the manipulation that was used had widespread effects throughout the corticomesolimbic circuit. However, a reduction in cocaine-induced cFos was observed in the NAc, but not AMYG, when activity of corticostriatal neurons was dampened (Kerstetter et al., 2016). This demonstrated the manipulation in cortical neurons did indeed affect the function of neurons in the NAc (ie. the direct target of these corticostriatal afferents). However, corticostriatal collaterals to the AMYG were not sufficient to modulate activity of AMYG neurons (Kerstetter et al., 2016). Nonetheless, this work demonstrates alterations in neurons downstream of those expressing hM4Di, suggesting more widespread effects

of the manipulation on circuit function. Therefore care must be taken when interpreting results from studies utilizing systemic CNO. These issues could be circumvented by direct application of CNO to hM4Di-expressing terminals via cannulas in the target region of interest (ie. NAc in these studies), but this method is much more invasive (Mahler et al., 2014). Nonetheless, an advantage of the systemic approach is that the manipulation maintains the intrinsic connectivity of the circuit, giving broader insight into function of the circuit as a whole.

Overall, the combination of chemogenetics and viral mediated gene transfer approaches provide a relatively simple, minimally invasive technique for reversible inactivation (or activation) of neurons. With the rise in the development of transgenic rats, chemogenetics paired with viral approaches could provide even better cell-type or spatial specificity of DREADD expression. Because GPCR are a common therapeutic target for pharmacological treatment of neuropsychological disorders, use of DREADD to probe circuit function could yield new insight into novel therapeutic targets for addiction (Bradley & Smith, 2016). Furthermore, because AAVs are already approved for use in humans this technique has the possibility of providing cell-type specific interventions for disorders such as addiction.

Corticostriatal work. This work was among the first reports to specifically probe the role of corticostriatal afferents in psychomotor sensitization to amphetamine. Consistent with previous literature, dampening activity of corticostriatal neurons blunted the development of amphetamine sensitization (Steketee & Kalivas, 2011; Vanderschuren & Kalivas, 2000; Wolf, 1998). While it is unlikely that this manipulation altered the expression of sensitization, a follow-up study to directly test this could be

completed where corticostriatal activity is reduced prior to the amphetamine challenge session. It was quite surprising that reducing corticostriatal activity during the development of sensitization did not alter the long-term persistent sensitized response to amphetamine, as prior work demonstrated lesions of PFC also block sensitized response during a challenge session (Li & Wolf, 2002; Wolf et al., 2003). My study utilized a lower dose of amphetamine for the challenge session (0.5 mg/kg) than that administered during sensitization (2 mg/kg). Thus it is possible the low dose was insufficient for me to detect an alteration in the long-term sensitized response following reduction in corticostriatal activity. Alternatively, PFC also projects to the ventral tegmental area, which is also a critical regulator of sensitization. Thus, it's possible that PFC projections to ventral tegmental area were responsible for the effect seen in the lesion studies.

However, the same manipulation did enhance conditioned responses related to the amphetamine injection. Several factors can contribute to a conditioned response associated with amphetamine injections, such as exposure to the drug-taking environment, interoceptive cues associated with the rewarding effects of amphetamine, and even the injection procedure itself. While I cannot control for the last two aspects, I could have tested the context-specificity (equipment limitations did not allow me to do this). A separate group of rats could have been sensitized in the home cage, and the challenge session carried out in a novel environment. If the environment were responsible for the enhanced conditioned response, then rats sensitized in the home cage with dampened corticostriatal activity would not have shown a conditioned response during the challenge session. Overall these results point to the idea that direct

modulation of NAc activity by cortical neurons is not crucial for amphetamine sensitization. In fact recent optogenetic studies have identified ventral hippocampus and basolateral amygdala inputs to NAc as important regulators of behavioral sensitization (Britt et al., 2012; MacAskill et al., 2014).

Next this work explored how corticostriatal afferents modulate aspects of cocaine-intake and cocaine-seeking. I demonstrated that corticostriatal afferents were not responsible for cocaine intake. However, the test used was a modified version of a progressive ratio test, which is intended to measure motivation for drug intake. Specifically, in order to accommodate CNO duration of action, test sessions were limited to 2 h. Due to this time limitation, some but not all rats reached the true breakpoint (ie. maximal number of lever presses rat is willing to perform to receive a single cocaine injection) during this session. Thus we were only able to measure cocaine intake and not motivation. Future studies should be performed in which corticostriatal activity is blunted for longer periods (ie. higher dose of CNO, multiple CNO injections, or delivery via an osmotic pump) to more clearly define whether corticostriatal neurons contribute to the motivation to self-administer cocaine. Furthermore, these studies were performed on rats that had achieved stable cocaine self-administration. It's possible that corticostriatal neurons contribute to the acquisition of cocaine self-administration. To test for this possibility, neuronal activity of corticostriatal neurons could have been reduced during each cocaine self-administration session. Nonetheless, dampening corticostriatal activity did not alter on-going cocaine intake, but did reduce cocaine-prime reinstatement, an effect consistent with optogenetic studies showing reduced cocaine, cocaine-plus-cue, and cue-induced

reinstatement (Ma et al., 2014; Stefanik et al., 2012; 2015). More importantly these results support the idea that relapse can be attributed to a hyperactive response in the prefrontal cortex in response to drugs and drug-associated stimuli (Feil et al., 2010; Goldstein & Volkow, 2011), making corticostriatal neurons a potential target for therapeutic interventions in relapse.

I also demonstrated that the DREADD manipulation during drug-taking had effects on subsequent responding to stimuli associated with cocaine intake. In particular, reducing corticostriatal activity during drug-taking impaired extinction and increased cocaine-primed reinstatement. This demonstrated the complex effects that an hM4Di manipulation during one phase of self-administration can have on subsequent phases. Without a GFP control group used in these studies, these effects would not have been detected. Nonetheless, care should be taken in interpreting these results. Similar to the sensitization data, it could be that reducing corticostriatal activity allowed for other inputs to drive the enhanced responding seen during the few extinction session and following a cocaine prime. Alternatively, activating of $G_{i/o}$ -signaling cascades may have longer lasting effects on neuronal function that are not solely due to a reduction in neuronal activity. For example, $G_{i/o}$ cascades can increase ERK/MAPK function, which can lead to long-term changes in plasticity (ie. days), which could lead to enhanced responsiveness to drug-associated cues. A method to avoid these long-term changes in circuit would have been to perform the corticostriatal manipulation during only a single aspect of self-administration or reinstatement. Future studies should be careful to use appropriate controls or utilize a within-subject design to avoid the possible long-term effects of hM4Di activation.

Overall this work identified a novel role of corticostriatal neurons in modulating the responsiveness to psychostimulant drugs and drug-associated stimuli. This work is consistent with the idea that extensive psychostimulant use leads to a hypoactive PFC at baseline but heightened PFC activation to both the drug and drug-related cues (Goldstein & Volkow, 2011). However, these studies utilized short access cocaine self-administration, a model that can provide insight into basic circuit function, but comes short in modeling compulsive aspects of addiction. Thus, it will be important to begin to understand whether corticostriatal neurons are important for compulsive drug use. Recently one study showed that corticostriatal neurons are important modulators of punishment-resistant cocaine seeking in rats (Chen et al., 2013). Furthermore, future studies can work to dissociate the role of specific classes of corticostriatal neurons. For example, cortical neurons can be split into two types that preferentially project to direct pathway (inratelencephalic) or indirect pathway (pyramidal tract) medium spiny neurons in the striatum (for review see Shepherd, 2013). Specifically targeting these two populations could give important insight into the influence of cortical glutamate on these two functionally distinct populations of NAc medium spiny neurons, which differentially regulate addiction.

Thalamostriatal work. While short access self administration paradigms have provided key insights into the circuitry associated with addiction, this model fails to fully capture the transition to compulsive drug use (Ahmed, 2012; Belin-Rauscent et al., 2016). In chapter 3, I present evidence that intermittent access to cocaine self-administration (IntA), which models a binge-like pattern of cocaine intake, can model key aspects of compulsive addiction, such as escalation of cocaine intake and

resistance to extinction. These results, in combination with others showing IntA leads to enhanced motivation for drug-taking and cocaine-primed reinstatement, strongly suggest IntA may be a better option for behavior studies as it more closely models compulsive addiction in an efficient manner (Martin-Garcia et al., 2014; Zimmer et al., 2012b). However, it remains to be determined whether drug-craving or drug-seeking behaviors are enhanced relative to short access and extended access cocaine self-administration. Furthermore these studies imposed a brief (4 second) timeout period between cocaine infusions. Evidence from studies that eliminate time-outs suggest that rats titrate the brain concentration of cocaine around a set-point, and that rats that have a higher frequency of cocaine infusions have exhibit higher levels of reinstatement (Martin-Garcia et al., 2014; Zimmer et al., 2012a). Future studies should consider removing the time-out in order to include analysis on the frequency of cocaine intake and how that relates to reinstatement behavior. Lastly, in my studies the levers were only extended into the chambers during drug available periods and were retracted for the time out. It would have been interesting to allow access to the levers during the drug unavailable period then perform analysis on drug-seeking during the time out period, with the goal of assessing whether or not drug-seeking during unavailable periods can predict relapse behaviors such as reinstatement. Prior studies have demonstrated that higher seeking during the time out periods correlates with higher reinstatement and resistance to punishment (Belin-Rauscent et al., 2016). Thus, much more work needs to go into validating IntA as a model of the transition to compulsive drug use.

The experiments that I performed in the MTN was the first study to utilize a reversible inactivation strategy to probe MTN function in cocaine relapse behaviors. I

demonstrate that dampening activity of MTN reduced both cue-induced and cocaine-primed reinstatement of drug-seeking, a finding consistent with other studies that have identified MTN in regulating context-induced reinstatement of cocaine and alcohol seeking (James & Dayas, 2013). These same regions have been implicated in regulating responses to cues associated with highly palatable food rewards (for review see Kelly et al., 2005), suggesting that MTN play a more general role in cue-motivated behaviors. In fact, a follow-up study is currently in progress where I dampen MTN activity during exposure to cues associated with palatable food intake to explore this idea. I am anticipating that this manipulation will blunt the reinstatement of cue-induced food seeking, similar to what was observed with drug cues. Regardless of the outcome of this experiment, the drug studies have already provided new insight into the role of MTN in relapse.

I extended these findings and demonstrated that dampening activity of anterior thalamostriatal neurons abolished cocaine-primed reinstatement but enhanced cue-induced reinstatement. I had no effect of my manipulation in posterior thalamostriatal neurons. These results demonstrate that specifically anterior thalamostriatal neurons are sufficient to account for MTN mediated aspects of cocaine-primed reinstatement, a mechanism that could be attributed to thalamic regulation of dopamine release in the NAc. While I provide did not examine this idea directly, one could use fast scan voltammetry in NAc to measure sub-second dopamine release in response to cocaine-associated cues. In the presence of CNO, dopamine release would be attenuated.

The effect on cue-induced reinstatement was surprising, and may be due to thalamic regulation of cholinergic interneurons. An alternative explanation is that cue-

induced reinstatement is regulated by different sets of thalamic efferents. I demonstrated MTN project strongly to PFC and AMYG, both of which are known to regulate cue-induced reinstatement. Thus, follow-up studies should explore the role of other sets of thalamic efferents in relapse. I have preliminary data (not presented in this thesis) that thalamic neurons projecting to AMYG do not regulate cue-induced reinstatement. However, a follow-up study should also define thalamocortical contributions to relapse. It is possible that I may have no effect of my manipulation in thalamic efferents on reinstatement. MTN also receive inputs from a variety of regions involved in arousal, perception, and emotion (Van der Werf et al., 2002). In fact, orexin and hypocretin expressing neurons originating in the hypothalamus have been shown to be important in context-induced reinstatement of alcohol seeking, or PFC neurons projecting to MTN may exert top down influences on motivated behaviors such as relapse (Haight & Flagel, 2014; Martin-Fardon & Boutrel, 2012). Regardless, this is the first work to demonstrate that thalamostriatal neurons regulate cocaine relapse and suggest thalamostriatal neurons may be a promising therapeutic target for preventing relapse.

Concluding remarks. This work has helped to establish how PFC and MTN afferents to NAc regulate addiction-related behaviors, such as psychomotor sensitization, drug-taking, and relapse. The NAc receives glutamatergic inputs from several regions, including PFC, MTN, AMYG, and HIPP. While recent optogenetic and chemogenetic studies have established roles of PFC, AMYG, and HIPP inputs to NAc in several addiction behaviors, none have assessed how MTN afferents regulate these behaviors. My work is the first to demonstrate the importance of thalamostriatal neurons

to reinstatement of cocaine-seeking. Furthermore, I identified a novel role of corticostriatal neurons in the associative processes that give incentive value to cocaine and cocaine-paired stimuli, and confirmed the role of these neurons in cocaine-primed reinstatement. Until this work, it has been difficult to make direct comparisons between studies on different NAc afferents due to variations in behavioral paradigms and technique parameters. However, the work in this thesis “filled in the gaps” by demonstrating a role of thalamostriatal neurons in regulating cocaine relapse, thus providing the last piece of information necessary to compare how different NAc glutamate sources regulate relapse.

Overwhelmingly optogenetic inhibition, optogenetic stimulation protocols that reverse cocaine-induced plasticity, or chemogenetic inhibition of any glutamatergic NAc afferent (ie. PFC, MTN, AMYG, and HIPPP) leads to a reduction in cocaine-seeking (for review Yager et al., 2015). The only exception are manipulations that specifically target infralimbic cortex projections to NAc shell; manipulations in this region have demonstrated enhanced cocaine-seeking (Ma et al., 2014; Pascoli et al., 2014). In this thesis, I also show evidence that anterior MTN neurons projecting to NAc, which primarily terminate in NAc shell, also enhance cue-induced reinstatement. Thus, while there may be regional differences in the overall role of glutamatergic neurotransmission during reinstatement (ie. shell manipulations enhance reinstatement whereas NAc or NAc core manipulations decrease relapse), chemogenetic or optogenetic interrogation of specific glutamatergic afferents into NAc all have the same effect on reinstatement behavior. Thus, this seems to suggest that the source of striatal glutamate may not be

particularly important in driving relapse and that generally altering glutamatergic tone within the NAc may be sufficient to prevent relapse.

However, recent anatomical studies have demonstrated that striatal glutamatergic afferents may preferentially target the two heterogeneously mixed populations of striatal medium spiny neurons (MSN), striatonigral direct pathway MSN (dMSN) and striatopallidal indirect pathway MSN (iMSN) (Britt et al., 2012; Lei et al., 2013; MacAskill et al., 2014; Pascoli et al., 2011; Shepherd, 2013; Wall et al., 2013). An imbalance in function between these two pathways has been hypothesized to mediate addiction and relapse (Lobo & Nestler, 2011; Yager et al., 2015). A few studies have begun to explore how specific NAc afferents regulate addiction-related changes in plasticity in dMSN and iMSN. Interestingly, electrophysiological studies have demonstrated post-synaptic changes in glutamatergic signaling in dMSN and iMSN not only depend on the source of glutamate, but the underlying mechanisms driving these changes in plasticity are also different based on the source of glutamate (Wolf, 2016; Yager et al., 2015). For example, withdrawal from cocaine self-administration resulted in enhanced glutamate signaling in dMSN but not iMSN, and dMSN changes were driven by inputs from PFC, but not AMYG or HIPPO (Pascoli et al., 2014). Work of this type is only beginning, and future research should explore input-specific manipulations in dMSN and iMSN. Furthermore, a more in-depth understanding of how cocaine alters neuronal activity and neurotransmitter release in the various NAc glutamatergic afferent regions themselves will be necessary to understand how the pattern and source of glutamate mediate alterations in drug induced plasticity that contribute to relapse.

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- Zimmer, B. A., Oleson, E. B., & Roberts, D. C. (2012b). The Motivation to Self-Administer is Increased After a History of Spiking Brain Levels of Cocaine, 37(8), 1901–1910.

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EDUCATION

2007 B.S. **University of Iowa**, Psychology (Honors)

POSITIONS

2011-2016 **University of Washington** Seattle, WA
Graduate Student
Seattle Children's Research Institute, Center for Integrative Brain
Research (CIBR)
Advised by Susan Ferguson
Defining corticostriatal and thalamic circuitry in addiction-related behavior

2007-2011 **University of Iowa Hospitals and Clinics** Iowa City, IA
Research Assistant III
Dept. of Psychiatry
Advised by John Wemmie
Role of acid-sensing ion channels in rodent models of psychiatric illness

2006-2007 **University of Iowa** Iowa City, IA
Undergraduate Honors Research
Dept. of Psychology
Advised by Joshua Rodefer
*Thesis: The spontaneously hypertensive rat as a model of attention-deficit
hyperactivity disorder (ADHD)*

2004-2007 **University of Iowa** Iowa City, IA
Undergraduate Researcher
Dept. of Internal Medicine and Howard Hughes Medical Institute
Advised by Michael Welsh and Margaret Price
Contribution of acid-sensing ion channels to pain and sensation

AWARDS AND HONORS

2015 Society for Neuroscience Trainee Professional Development Award
2015 University of Washington Graduate School Fund for Excellence and
Innovation Travel Award
2012, 2013 NSF Graduate Research Fellowship, honorable mention

- 2006 University of Iowa Student Government Undergraduate Research Grant, \$500
- 2002 Horn Memorial Scholarship Fund Recipient

PEER-REVIEWED PUBLICATIONS

- Kerstetter, K.A.*, **Wunsch, A.M.***, Nakata, K.G., Donckels, E., Neumaier, J.N., & Ferguson, S.M. (2016) Corticostriatal afferents modulate responsiveness to psychostimulant drugs and drug-associated environmental stimuli. *Neuropsychopharmacology*, 41(4): 1128-1137. PMID: 26289144
[*indicates co-first authorship]
- Yager, L.M., Garcia, A.F., **Wunsch, A.M.**, & Ferguson, S.M. (2015) The ins and outs of the striatum: Role in drug addiction. *Neuroscience*, 301: 529-541. PMID: 26116518.
- Darvas, M., **Wunsch, A.M.**, Gibbs, J., & Palmiter, R. (2014) Dopamine dependency for acquisition and performance of Pavlovian conditioned response. *Proceedings of the National Academy of Sciences*, 111(7): 2764-9. PMID: 24550305.
- Stewart, A., Maity, B., **Wunsch, A.M.**, Meng, F., Wu, Q., Wemmie, J.A., & Fisher, R.A. (2014) Regulator of G-protein signaling 6 (RGS6) promotes anxiety and depression by attenuating serotonin-mediated activation of the 5-HT_{1A} receptor-adenylyl cyclase axis. *FASEB J.*, 28(4): 1735-44. PMID: 24421401.
- Urbano, F., Lino, N., Gonzalez-Inchauspe, C., Gonzalez, L., Colettis, N., Vattino, L., **Wunsch, A.**, Wemmie, J., & Uchitel, O. (2014) Acid sensing ion channels 1a (ASIC1a) inhibit neuromuscular transmission in female mice. *American Journal of Physiology-Cell Physiology*, 306(4): C396-406. PMID: 24336653.
- Vralsted, V.C., Price, M.P., Schnizler, M., **Wunsch, A.M.**, Ziemann, A.E., Welsh, M.J., & Wemmie, J.A. (2011). Expressing acid-sensing ion channel 3 in the brain alters acid-evoked currents and impairs fear conditioning. *Genes, Brain, & Behavior*, 10(4): 444-50. PMID: 21324060.
- Render, J.A., Howe, K.R., **Wunsch, A.M.**, Guionaud, S., Cox, P.J., & Wemmie, J.A. (2010) Histologic examination of the eye of acid-sensing ion channel 1a knockout mice. *International Journal of Physiology, Pathophysiology, & Pharmacology*, 2(1): 69-72. PMID: 21383900.
- Ziemann, A.E., Allen, J.E., Dahdaleh, N.S., Drebot, I.I., Coryell, M., **Wunsch, A.M.**, Lynch, C.M., Faraci, F.M., Howard III, M.A., Welsh, M.J., & Wemmie, J.A. (2009) The amygdala is a chemosensor that detects hypercarbia and acidosis to elicit fear behavior. *Cell*, 139: 1012-1021. PMID: 19945383.
- Coryell, M.W., **Wunsch, A.M.**, Haenfler, J.M., Allen, J.E., Schnizler, M., Ziemann, A.E., Cook, M.N., Dunning, J.P., Price, M.P., Rainier, J.D., Liu, Z., Light, A.R., Langebehn, D.R., & Wemmie, J.A. (2009) Acid-sensing ion channel-1a in the

amygdala, a novel therapeutic target in depression-related behavior. *The Journal of Neuroscience*, 29(17): 5381-5388. PMID: 19403806.

Coryell, M.W., **Wunsch, A.M.**, Haenfler, J.M., Allen, J.E., McBride, J.L., Davidson, B.L., & Wemmie, J.A. (2008) Restoring Acid-Sensing Ion Channel-1a in the amygdala of knockout mice rescues fear memory but not unconditioned fear responses. *The Journal of Neuroscience*, 28(51): 13738-13741. PMID: 19091964.

SEMINARS AND PRESENTATIONS

- 2015 Oct. Society for Neuroscience Conference, New Findings in Neural Mechanism of Addiction Nanosymposium (Chicago, IL)
- 2015 Jun. Seattle Children's Research Institute, Office of Animal Care Seminar (Seattle, WA)
- 2015 May University of Washington, Neuroscience Spring Symposium (Seattle, WA)
- 2015 Mar. Seattle Children's Research Institute, Center for Integrative Brain Research Lunch Series (Seattle, WA)

POSTER PRESENTATIONS AND ABSTRACTS

(italics indicate presenting author)

Wunsch, A.M., Donckels, E.A., Yager, L.M., Neumaier, J.F., & Ferguson, S.M.

Determining the contribution of nucleus accumbens glutamatergic afferents during addiction-related behaviors using Gi/o-coupled DREADDs. Society for Neuroscience Meeting, Oct. 2015

Wunsch, A.M., Donckels, E.A., Neumaier, J.F., & Ferguson, S.M. Resolving the contribution of nucleus accumbens glutamatergic afferents during amphetamine sensitization using Gi/o-coupled DREADDs. Society for Neuroscience Meeting, Nov. 2014

Wunsch, A.M., & Ferguson, S.M. Role of G_{i/o}-signaling cascades in striatal subregions during delay discounting. Society for Neuroscience Meeting, Nov. 2013.

Wunsch, A.M., Allen, J.E., Zenner, K., & Wemmie, J.A. Carbon dioxide & acidosis enhance fear memory consolidation through ASIC1a. Society for Neuroscience Meeting, Nov. 2010

Wunsch, A.M., Allen, J.E., Zenner, K., & Wemmie, J.A. Effects of altering brain pH on fear conditioning. University of Iowa Research Week. Mar. 2010

Wunsch, A.M., Price, M.P., Lawrence, I., Welsh, M.J., & Wemmie, J.A. ASIC2 disruption increases carbon dioxide fear-like responses. Society for Neuroscience Meeting, Nov. 2009

Wemmie, J.A., Coryell, M., **Wunsch, A.**, Haenfler, J., Allen, J., Schnizler, M., Ziemann, A., Cook, M., Dunning, J., Price, M., Rainier, J., Liu, Z., Light, A., & Langbehn, D. Acid-sensing ion channel-1a in the amygdala, a novel therapeutic

target in depression-related behavior. Society for Neuroscience Meeting, Oct. 2009

Coryell, M.W., **Wunsch, A.M.**, Allen, J.E., Ziemann, A.E., Price, M.P., Langebehn, D.R., & Wemmie, J.A. Acid-sensing ion channel-1a in the amygdala, a novel therapeutic target in depression-related behavior. University of Iowa Carver College of Medicine Research Week, Mar. 2009

Lobas, M.A., **Wunsch, A.M.**, & Wemmie, J.A. ASIC1a contributes to fear expression and fear memory. Society for Neuroscience Meeting, Nov. 2008

Wunsch, A.M. & Rodefer, J.S. Cognitive and behavioral characteristics of the Spontaneously Hypertensive Rat (SHR)-a model of ADHD? Society for Neuroscience Meeting, Nov. 2007

Wunsch, A.M., Nguyen, T., Guynn, L., & Rodefer, J. Effects of 5-HT6 antagonists on attenuating PCP-induced cognitive deficits in a rodent set-Shifting (ED/ID) task. Society for Neuroscience Meeting, Nov. 2006.

TEACHING

2013 University of Washington, NBIO 302 Introduction to Systems and Behavioral Neuroscience: Teaching Assistant and Laboratory Instructor

MENTORING

2014-2016 Calvin Le, undergraduate researcher, Biochemistry and Neurobiology major at University of Washington

2012-2014 Elizabeth Donckels, Mary Gates Foundation fellow & undergraduate researcher, Neurobiology major at University of Washington

PROFESSIONAL ACTIVITIES

2015-2016 Scientific Content Editor, *Grey Matters* Journal

OUTREACH AND COMMUNITY INVOLVEMENT

2012-2016 Brain Awareness Week, volunteer

2012-2015 Pacific Life Sciences Weekend, Pacific Science Center, volunteer

2012 Seattle Public Libraries Lecture on comparative anatomy for adults

2010 Geriatric Center Volunteer in Cusco, Peru

2003-2005 University of Iowa Women in Science and Engineering Mentor