Cortical and subthalamic circuits in the regulation of addiction-like behaviors

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Abstract

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Addiction is a debilitating and complex neuropsychiatric disorder that generates substantial medical, social, monetary, and emotional costs for both addicted individuals and society. In spite of these costs, relapse rates remain high and effective interventions lacking, attributable in part to our limited understanding of the complex pathophysiology and neural substrates underlying addiction. The corticolimbic circuitry which underlies many of the maladaptive behaviors associated with addiction is a complex and interwoven network. Under normal conditions, this network helps regulate a wide array of motor, associative, and affective behaviors, allowing us to shape our actions adaptively in response to environmental rewards, dangers, and other stimuli. However, the use of addictive drugs initiates a broad cascade of intra- and extracellular changes to this circuitry, which are believed to underlie the transition from controlled, volitional drug use to the uncontrolled, compulsive drug abuse that defines addiction. The interconnectivity

of addiction circuitry presents a significant challenge for studies seeking to explore how addiction emerges from drug-induced neuroadaptations, but recent advances in virus-mediated chemogenetic manipulation have allowed targeted examination of discrete neuronal circuits in ways not previously possible, allowing us to examine how specific components of the corticolimbic network contribute to drug addiction.

The overarching goal of this dissertation was to leverage chemogenetic tools to investigate how corticostriatal and subthalamic circuits, critical components of the corticolimbic network, regulate addiction-like behaviors in rodents, including locomotor sensitization to amphetamine as well as self-administration and seeking of cocaine. The first chapter establishes the current state of knowledge surrounding cortical and subthalamic involvement in drug addiction. The second chapter demonstrates how inhibition of neuronal projections from medial prefrontal cortex to the nucleus accumbens transiently attenuates the development of amphetamine sensitization while also enhancing conditioned responding to drug-associated cues, slowing extinction of cocaine self-administration, and enhancing drug-primed reinstatement of drug seeking. The third chapter describes how stimulation of the subthalamic nucleus dramatically blocks the development of amphetamine sensitization, while inhibition only transiently enhances induction of sensitization. It also describes how inhibition of subthalamic afferents from the ventral pallidum and prelimbic cortex both attenuate conditioned responding to drug-associated procedures, with only the prelimbic projection significantly attenuating the persistence of sensitization and neither altering its induction, suggesting a complex role for the subthalamic nucleus in larger addiction circuitry.

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Chapter 1

Introduction

*Parts of this introduction were published with minor reformatting as part of a peer-reviewed review article in *Pharmacology Biochemistry and Behavior*. Co-authors of the final manuscript included Aaron F. Garcia and Susan M. Ferguson. The full citation is as follows:

Garcia AF, Nakata KG, Ferguson SM (2017). Viral strategies for targeting cortical circuits that control cocaine-taking and cocaine-seeking in rodents. *Pharmacology Biochemistry and Behavior* 174: 33–41.

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

1.1 Psychostimulant Addiction

Addiction to psychostimulant drugs is a debilitating neuropsychiatric disorder that carries with it substantial medical, social, monetary, and emotional costs for both addicted individuals and society. In spite of these costs and initiatives intended to address them, overall psychostimulant misuse remains common. Based on data from the National Survey on Drug Use and Health (2016), approximately 1.9 million Americans misuse cocaine, 1.7 misuse prescription stimulants, and 700,000 misuse methamphetamine. Addiction to these drugs, which is differentiated from mere use by a loss of control to the point of compulsive drug seeking and taking despite adverse consequences, also affects significant portions of the population. These same survey data suggest an estimated 867,000 Americans meet the clinical criteria for having a cocaine use disorder, with another 684,000 meeting criteria for methamphetamine use disorder and 540,000 meeting criteria for prescription stimulant use disorder. Not captured in these numbers are the many friends, family members, and others also impacted by these addictions in some form.

In spite of these consequences, relapse rates remain high, and pharmacological interventions are lacking, attributable in part to an inadequate understanding of the complex pathophysiology and neural substrates underlying addiction. Psychostimulant drugs exert their complex combination of euphoric, rewarding, and aversive effects in part by elevating synaptic concentrations of dopamine through inhibition of dopamine reuptake (cocaine and amphetamine) and increased dopamine release (amphetamine), which in turn initiates a cascade of changes to corticolimbic circuitry (Baik, 2013; Berridge and Arnsten, 2013; Everitt *et al*, 2008; Nestler, 2001). This corticolimbic perturbation triggered

by psychostimulants is thought to underlie much of the transition from controlled drug use to the compulsive use associated with addiction (Berke and Hyman, 2000; Lobo and Nestler, 2011; Lüscher and Malenka, 2011; Moussawi *et al*, 2011; Nestler, 2001; van Huijstee and Mansvelder, 2014; Yager *et al*, 2015), with many of these changes centered around the striatum, a critical interface within this circuitry (Lobo and Nestler, 2011; Nestler, 2013; van Huijstee and Mansvelder, 2014; Yager *et al*, 2015).

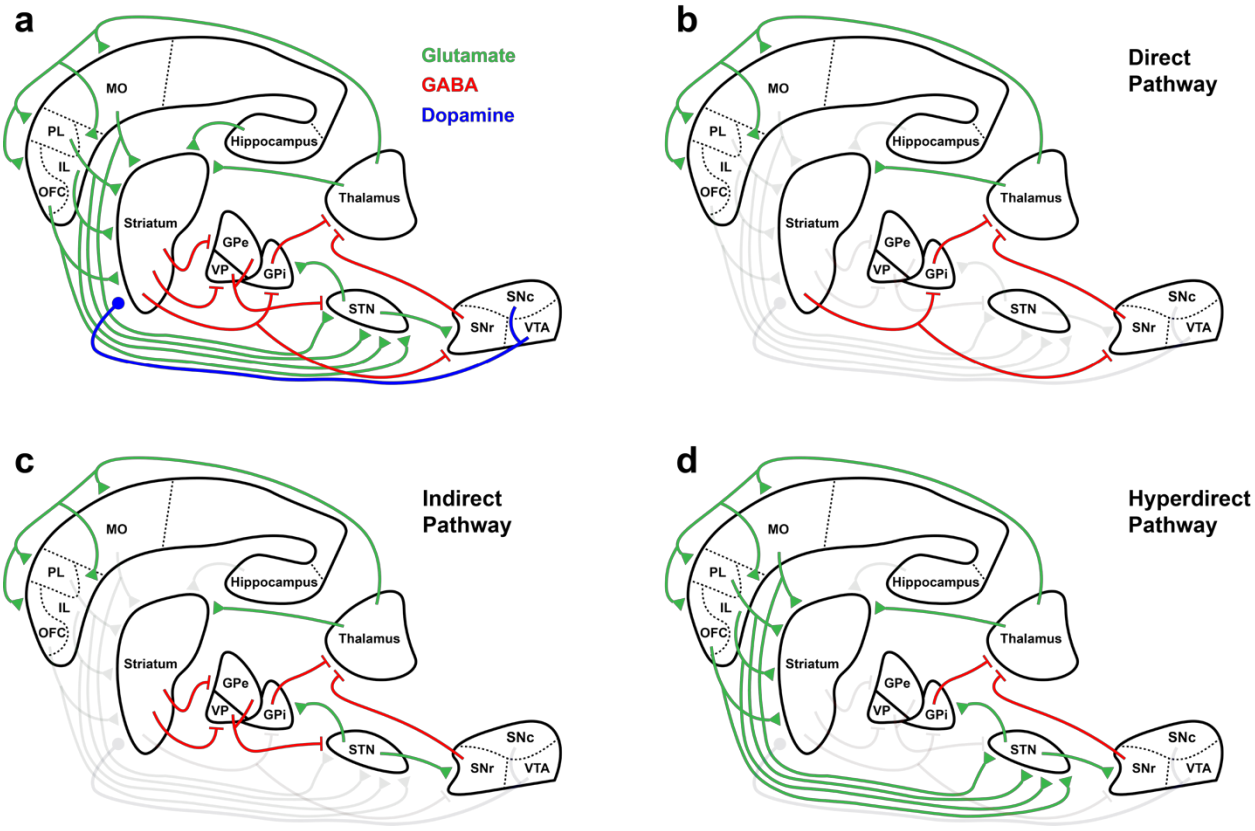

Figure 1.1. Corticolimbic circuits. (**a**) Simplified illustration of basic corticolimbic circuitry including corticostriatal-basal ganglia-thalamocortical loops. (**b**) Illustration highlighting components of the direct striatal pathway, which generally promotes behavioral execution. (**c**) Illustration highlighting components of the indirect striatal pathway, which generally inhibits behavioral execution. (**d**) Illustration highlighting components of the hyperdirect or corticosubthalamic pathway, the function of which remains understudied, but may involve promotion of behavioral flexibility or gating through rapid top-down behavioral inhibition that bypasses much of the indirect striatal pathway.

The striatum consists primarily of GABAergic medium spiny projection neurons (MSNs) that integrate a wide variety of inputs, including glutamatergic inputs from the cortex and dopaminergic inputs from the ventral tegmental area and substantia nigra (Britt *et al*, 2012; Finch, 1996; Groenewegen *et al*, 1999; Phillipson and Griffiths, 1985; Swanson, 1982; Yager *et al*, 2015) (Figure 1.1a). Classically, striatal MSNs then send outputs via two primary pathways: the direct pathway (Figure 1.1b), in which MSNs expressing dopamine D1 receptors, dynorphin, and substance P directly project to output nuclei of the basal ganglia (internal globus pallidus and substantia nigra), and the indirect pathway (Figure 1.1c), in which MSNs expressing dopamine D2 receptors and enkephalin multisynaptically relay information to basal ganglia output nuclei via the external globus pallidus/ventral pallidum and subthalamic nucleus (STN) (Calabresi *et al*, 2014; Gerfen and Surmeier, 2011; Lobo and Nestler, 2011; Wall *et al*, 2013; Yager *et al*, 2015), with some notable exceptions to pathway segregation (Fujiyama *et al*, 2011). These two output pathways are generally thought to act as balanced, opposing forces in guiding behavior, with direct pathway activity promoting behavioral execution and indirect pathway activity inhibiting behaviors (Lobo and Nestler, 2011; Wall *et al*, 2013; Yager *et al*, 2015), though these pathways also exhibit more complex interactions not entirely accounted for by this simplified model (Calabresi *et al*, 2014). Dysregulation of these pathways and the balance of their activity is thought to contribute to a variety of neuropsychiatric disorders, including addiction, making the circuitry along these pathways the subject of intense study (Lobo and Nestler, 2011; Smith and Graybiel, 2013; Volkow *et al*, 2013; Yager *et al*, 2015). A third pathway, the corticosubthalamic or hyperdirect pathway (Figure 1.1d), has frequently been left out of the traditional direct and indirect

striatal pathway model in spite of early anatomical studies identifying these projections (Canteras *et al*, 1990; Nambu *et al*, 1996; Romansky *et al*, 1979) and consists of direct glutamatergic projections from cortical regions to the STN, shortcutting the early components of the indirect striatal pathway. This hyperdirect pathway remains poorly characterized, though it may be involved in the gating of behavioral programs (Cavanagh *et al*, 2011) or promotion of behavioral flexibility through rapid top-down behavioral inhibition (Baker and Ragozzino, 2014), ideas discussed in greater depth later in this chapter and in Chapters 3 and 4. While a comprehensive review of this neurocircuitry in addiction is beyond the scope of this work (see Lobo and Nestler, 2011; Smith and Graybiel, 2013; Volkow *et al*, 2013; Yager *et al*, 2015 for more extensive review), the studies to follow will highlight the role of two essential components within this corticolimbic system: corticostriatal projections from medial prefrontal cortex (mPFC) and the STN, including STN afferents from the ventral pallidum (via the indirect pathway) and prelimbic cortex (via the hyperdirect pathway).

1.2 Viral Strategies for Targeting Addiction Circuitry

Investigating the complex neurocircuitry involved in addiction has been accomplished by a wide variety of methods, including pharmacological manipulation and lesioning, yet few tools have proven as powerful in dissecting the functions of discreet circuits as cell-specific, virally-mediated genetic manipulation of neuronal activity. Specifically, optogenetic and chemogenetic methods allow reversible, bidirectional modulation of targeted neurons through expression of light- and designer ligand-sensitive proteins respectively, and both have been widely adopted for the study of addiction

neurocircuitry. Optogenetics utilizes engineered light-sensitive proteins to reversibly manipulate neuronal activity on fast, millisecond timescales, with neuronal excitation typically achieved via the cation channel channelrhodopsin-2 and inhibition achieved via the chloride pump halorhodopsin or proton pump archaerhodopsin (Allen *et al*, 2015; Boyden *et al*, 2005; Deisseroth, 2015; Fenno *et al*, 2011; Mattis *et al*, 2011). This tight temporal resolution of optogenetic manipulation has proven valuable for studying how circuit activity and firing patterns modulate discrete behavioral actions, including the self-administration of drugs. Unlike optogenetics, chemogenetic tools utilize pharmacological methods to manipulate cellular activity through engineered proteins. In particular, DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) are widely used to reversibly modulate neuronal activity, commonly using a G_q- or G_s-DREADD (hM_3Dq and rM_3Ds) to stimulate neurons and a $G_{i/o}$-DREADD (hM_4Di or kappa-opioid receptor derived KORD) to inhibit them (Armbruster *et al*, 2007; Burnett and Krashes, 2016; Roth, 2016; Smith *et al*, 2016; Urban and Roth, 2015; Vardy *et al*, 2015). These designer receptors are activated through application of specialized ligands (clozapine-N-oxide (CNO), DREADD agonist 21, salvinorin B) either systemically or locally to DREADD-expressing cell populations and trigger signaling changes via the G-protein signaling cascades associated with a given DREADD variant, with some causing modulation of ion channels, such as inward rectifying potassium channels. The slower kinetics of chemogenetic manipulations often prevent pairing with rapid behavioral actions as is possible with optogenetics, but are particularly appropriate when neuronal modulation is desired for longer durations (such as a full behavioral testing session). While the engagement of G-protein signaling cascades allows physiologically-relevant

amplification of the desired manipulation, these cascades can also trigger complex cellular effects beyond excitation or inhibition, including through β-arrestin signaling. In addition, off-target effects of DREADD ligands are possible at higher doses (Gomez *et al*, 2017; MacLaren *et al*, 2016; Manvich *et al*, 2018), particularly with clozapine and CNO, which is now known to be converted into clozapine *in vivo* (Gomez *et al*, 2017), requiring careful use of appropriate controls when designing DREADD studies.

Finally, the targeting of specific cell types or neuronal projections is possible when using both chemogenetic and optogenetic tools. This is often accomplished through the use of selective genetic promoters, Cre-recombinase (Cre-)-lox recombination, and direct stimulation of axon terminals through local application of light or DREADD ligands. A common approach utilized by studies reviewed in the following section involves use of the CaM kinase II alpha (CaMKIIα) promoter to preferentially target excitatory pyramidal neurons of the cortex. However, it should be noted that CaMKIIα-driven viral expression has been observed in other cortical neuron populations, including some parvalbumin-positive cortical neurons, suggesting CaMKIIα promoters do not exclusively target excitatory pyramidal cells (Watakabe *et al*, 2015). Additionally, several studies, including work reviewed in the following section, as well as experiments described in Chapters 2 and 3, utilize (Cre-)-lox recombination to target specific neuronal projections with opto- or chemogenetic manipulation. Briefly, expression of a Cre-dependent DREADD or optogenetic construct with a double-floxed inverse open reading frame (DIO) (e.g., AAV8-hSyn-DIO-hM$_4$D$_i$-mCherry) is dependent on co-expression of Cre, which can be limited by cell type using specialized Cre-expressing transgenic animal lines or to specific projections through infusion of retrogradely transported Cre-expressing virus (e.g., CAV2-

Cre) into a downstream target, such that only cells projecting to that target are transfected and express Cre. In this way, only cells both transfected with the Cre-dependent DREADD or optogenetic construct and projecting to the downstream target of interest express both Cre and the DREADD or optogenetic construct, limiting experimental manipulation to that specific projection.

1.3 The Prefrontal Cortex and Addiction

The prefrontal cortex (PFC) regulates a wide range of cognitive, perceptual, emotional, and motivational processes, and dysregulation of these processes has been implicated in numerous neuropsychiatric disorders, including addiction. In particular, the PFC appears to play a critical role in the formation and maintenance of addictive behavior to drugs through its regulation of salience attribution, drug-associated memory expression, decision making, and inhibitory control, with important differences in function between cortical subdivisions (Goldstein and Volkow, 2011; Hogarth *et al*, 2013; Kalivas, 2009; Peters *et al*, 2013; Van den Oever *et al*, 2010; Volkow and Morales, 2015). The purpose of this review is to provide a brief primer on the role of the PFC in addiction based on studies utilizing rodent behavioral models in concert with viral-vector strategies for manipulating PFC neurocircuitry.

Research exploring the role of the PFC in psychostimulant addiction has largely focused on the mPFC, which has historically been thought of as a critical region for inhibitory control (Goldstein and Volkow, 2011; Kalivas, 2009). The mPFC becomes hypoactive in both humans (Goldstein and Volkow, 2011) and rodents (Chen *et al*, 2013) following chronic drug exposure, while also displaying increased activity following

exposure to drug-associated cues (Goldstein and Volkow, 2011; Kalivas, 2009; McFarland and Kalivas, 2001). The mPFC can be subdivided into two regions, the dmPFC (i.e., the anterior cingulate and dorsal aspect of prelimbic cortex) and the vmPFC (i.e., the ventral aspect of prelimbic cortex and the infralimbic cortex) (Heidbreder and Groenewegen, 2003; Moorman *et al*, 2015; Vertes, 2004), which have different roles in addiction-related behaviors in addition to having distinct patterns of connectivity. Specifically, activation of the dmPFC is generally thought to promote drug seeking, whereas activation of the vmPFC can either promote or inhibit drug seeking depending on experimental parameters, including the type of drug, whether extinction training occurs, and targeting of the manipulation (Koya *et al*, 2009; McLaughlin and See, 2003; Peters *et al*, 2008; Rogers *et al*, 2008; Van den Oever *et al*, 2013; Willcocks and McNally, 2013). Finally, the orbitofrontal cortex (OFC) is thought to mediate salience attribution and valuation of outcomes, such that drug-induced OFC dysfunction may limit behavioral flexibility and contribute to the habitual behavior associated with addiction (Lucantonio *et al*, 2014; Schoenbaum and Shaham, 2008; Volkow and Morales, 2015). Although these studies provide a general framework for the contribution of each of these regions of the PFC in addiction-related behaviors, it is important to note that there are significant discrepancies in the existing literature with respect to the functional subdivisions that are described here that render comparisons across some studies difficult. In particular, there is large variance between studies in whether the targeting of manipulations extended across most of the mPFC, were restricted to the dorsal or ventral portion, or were isolated to a specific subregion (e.g., prelimbic vs infralimbic cortex). This distinction is critical given that subdivisions of the mPFC, such as prelimbic and infralimbic cortex, have

important differences in their broader connectivity (Vertes, 2004) and especially function, which we demonstrate later in this review. For the purpose of this review, if viral expression occurred in both dmPFC and vmPFC, then we will refer to these studies as having targeted mPFC, with the caveat that cortical territories shown to have distinct functions are simultaneously targeted in this case. In addition, we will separate studies that targeted dmPFC from those that targeted prelimbic cortex, since the prelimbic studies would also have included some aspects of vmPFC. However, the studies targeting infralimbic cortex will be included in the vmPFC section, but a notation will be made if they were confined to the infralimbic portion of vmPFC. Finally, studies utilizing both rat and mouse models are included in this review, and while both the functional and anatomical subdivisions are thought to be similar between species, considerable disagreement remains over how to best compare cortical subdivisions across rodent, and especially non-rodent, species (Laubach et al, 2018). Addressing these ambiguities, such as through standardization based on curvature of the corpus collosum (Laubach et al, 2018), is a matter of ongoing discussion within the larger neuroscience community and is, for now, beyond the scope of this review.

dmPFC

The role of the dmPFC in addiction-related behaviors has not been studied using viral-mediated gene transfer techniques. However, optical inhibition of dmPFC using halorhodopsin has been shown to attenuate stress-induced reinstatement of food-seeking in rats (Calu *et al*, 2013), suggesting that the dmPFC promotes reward-seeking.

Prelimbic cortex

Chronic exposure to cocaine can trigger a reduction in baseline mPFC activity in addicted individuals (Goldstein and Volkow, 2011). A similar reduction in neuronal excitability has been observed in rats after prolonged cocaine exposure, and this reduction is especially pronounced in rats that show compulsive drug-taking (e.g., continue to seek cocaine when it is paired with foot shock) (Chen *et al*, 2013). Optogenetic manipulation of the prelimbic cortex has been used to study the role of this hypoactivity in the loss of inhibitory control. In particular, using AAVs expressing channelrhodopsin and halorhodopsin for neuronal excitation and inhibition, respectively, it was found that optical excitation of prelimbic cortex neurons reduced compulsive (i.e., shock-resistant) cocaine intake whereas optical inhibition of these neurons enhanced compulsive intake (Chen *et al*, 2013). These results suggest that PFC hypoactivity can, in fact, trigger a loss of inhibitory control over drug consumption. Similarly, photoinhibition of prelimbic cortical neurons with archaerhodopsin was found to enhance cocaine self-administration (Martín-García *et al*, 2014). However, this manipulation also reduced cocaine-primed reinstatement among rats that had undergone a high-frequency cocaine intake schedule (Martín-García *et al*, 2014), as did optical inhibition of prelimbic cortex using halorhodopsin (Stefanik *et al*, 2013). Taken together, these results suggest a more complex role of the PFC, where a reduction in activity of this region can either promote or inhibit drug-taking and drug-seeking behaviors depending on a number of factors including the presence of cocaine during testing, phase of the experimental paradigm (e.g., training, reinstatement), or pattern of drug use.

vmPFC

Similar viral targeting methods have also been used to study the role of vmPFC in drug-taking and drug-seeking behaviors. For example, chemogenetic activation of vmPFC with a G_q-DREADD attenuated cue-induced, but not cocaine-primed, reinstatement of cocaine seeking after extinction, yet had no effect on reinstatement after an equivalent period of drug abstinence without extinction training (Augur *et al*, 2016). This suggests that extinction-dependent plasticity in the vmPFC is necessary for vmPFC regulation of cue-driven cocaine seeking, possibly through the reconciliation of opposing cue-outcome associations or by preventing neuroadaptations that underlie the incubation of cocaine craving. However, vmPFC control over conditioned cocaine seeking may also be time dependent, as optical stimulation of vmPFC neurons using channelrhodopsin was found to facilitate the extinction of a cocaine conditioned place preference (CPP) after 3 weeks of drug abstinence, but there was no effect of the manipulation when extinction testing was done immediately after conditioning (Van den Oever *et al*, 2013). Similarly, optical inhibition of vmPFC neurons using halorhodopsin blocked extinction of a CPP memory to cocaine after a 3-week drug withdrawal; however, vmPFC inhibition using Cre-inducible halorhodopsin in transgenic C57BL/6 CaMKII::Cre mice was also able to attenuate the expression of a cocaine CPP 24 h after conditioning (Van den Oever *et al*, 2013). This work suggests that, during periods of drug abstinence, the circuitry underlying cocaine seeking may reorganize such that the vmPFC transitions from being necessary for the expression of drug-seeking to facilitating the extinction of drug-seeking when drug-associated cues no longer signal drug availability. Furthermore, the infralimbic region of the vmPFC may also play a role in how stressors influence the extinction of drug-seeking.

Exposure to inescapable tailshock stress was found to reduce the extinction of cocaine-seeking behavior, whereas exposure to escapable tailshock (i.e., shock was avoided by turning a wheel) accelerated extinction, and this facilitation was blocked by optical silencing of infralimbic neurons using halorhodopsin (Baratta *et al*, 2015). This work suggests that not only can controllability over a stressor influence how stress affects drug-seeking, but this process is regulated by the infralimbic cortex. Overall, these findings highlight a critical role for the vmPFC in extinction memory of cocaine, but also suggest that the role of the vmPFC is dependent on both the timing and duration of different phases of the experimental paradigm, particularly for drug abstinence and extinction training.

OFC

The role of the OFC in behaviors related to addiction has also been studied using viral-mediated optogenetic and chemogenetic tools. Since the OFC is thought to regulate salience attribution and valuation of rewarding outcomes, one behavioral approach used to study this region is to test for Pavlovian over-expectation. Rats trained to self-administer cocaine show a deficit in Pavlovian over-expectation such that distinct conditioned stimuli fail to elicit a summated response when compounded. This deficit can be reversed through optical excitation of OFC neurons using channelrhodopsin (Lucantonio *et al*, 2014). This finding indicates that OFC dysregulation after cocaine exposure may impair the value representation of instrumental outcomes, or more specifically, impair the ability to generate value representations of novel outcomes flexibly and based on prior knowledge or insight into similar outcomes that were previously experienced. Activation of medial OFC neurons with a G_s-DREADD was shown to

enhance sensitivity to devaluation of a food reward, while targeted knockdown of *Bdnf* in the medial OFC of floxed-*Bdnf* mice with Cre-expressing lentivirus blocked sensitivity to devaluation, suggesting that the OFC also plays a role in flexible responding to natural rewards and that this function is dependent on *Bdnf* signaling (Gourley *et al*, 2016). Finally, the OFC has also been implicated in regulating compulsive (i.e., punishment-resistant) reward-seeking as chemogenetic inhibition of OFC neurons using a $G_{i/o}$-DREADD decreases VTA self-stimulation when it is paired with foot shock. In addition, neuronal activity and excitability are enhanced in the lateral OFC following VTA self-stimulation paired with foot shock only in punishment-resistant mice (Pascoli *et al*, 2015). These results suggest that OFC inhibition could impair the updating of reward valuation of VTA self-stimulation such that the negative value of noxious shock was not fully integrated once introduced. Together, these studies indicate that OFC hypoactivity in addicted individuals may disrupt the creation of flexible value representations and promote compulsive, punishment-resistant behaviors that are characteristic of addiction.

1.4 The Subthalamic Nucleus and Addiction

The subthalamic nucleus (STN) is a relatively small subcortical nucleus and component of the basal ganglia system composed almost entirely of glutamatergic projection neurons, with a small population of GABAergic interneurons (Lévesque and Parent, 2005), long thought to function as a relay within the cortico-basal ganglia-thalamocortical loop (Canteras *et al*, 1990; Groenewegen and Berendse, 1990; Kita and Kitai, 1987; Temel *et al*, 2005). Further study began to uncover a critical role for the STN in the regulation of motor functions and highlighted its involvement in movement disorders

like Parkinson's disease (PD) (Albin *et al*, 1989). In particular, reduction of STN activity through lesions or high frequency stimulation (HFS) was found to improve parkinsonian symptoms (Limousin *et al*, 1995), and STN deep brain stimulation (DBS) is now commonly used to treat symptoms of advanced PD (Bari *et al*, 2015; Benabid *et al*, 2009).

This growing interest in STN regulation of motor functions, along with clinical application of STN DBS for PD, soon led to the identification of more cognitive functions not previously examined in STN literature. Studies revealed that STN lesions in PD-like 6-hydroxydopamine (6-OHDA) lesioned rats substantially increase the number of premature responses during a simple reaction time (RT) task, potentially attributable to an attentional deficit or disinhibition (Baunez *et al*, 1995). Similar impairments are observed using a more complex 5-choice serial RT task (5-CSRTT), where STN lesions markedly reduce task accuracy (Baunez and Robbins, 1997). In addition, local microinfusion of either APV (an NMDA receptor antagonist) or muscimol (a $GABA_A$ receptor agonist) into the STN also reduces 5-CSRTT accuracy, though these treatments have opposing effects on premature responding (Baunez and Robbins, 1999).

The role of the STN in motivational processes and addiction-related behaviors was later explored through a series of studies examining food and cocaine self-administration, conditioned place-preference (CPP), and locomotor sensitization. Those studies also began to generate diverging experimental outcomes, highlighting the complex role of the STN in these behaviors. For instance, cocaine-induced c-Fos mRNA expression, often interpreted as a proxy for neuronal activity, in the STN increases in parallel with locomotor sensitization to cocaine, suggesting that the STN may contribute to the psychomotor activating effects of cocaine (Uslaner *et al*, 2003). Excitotoxic STN lesions appear to

increase motivation for, and salience of, food rewards while decreasing motivation for, and salience of, cocaine and having no effect on the primary reinforcing properties of either reward (Baunez *et al*, 2005). This suggests that the STN might differentially modulate the processing of distinct rewards, and similar alterations of motivation and reward salience have been achieved using STN HFS (Rouaud *et al*, 2010). Similarly, pharmacological inhibition of STN with local microinfusion of the $GABA_A$ agonist muscimol reduces cocaine self-administration when consumption requires high-, but not low-, effort, while also reducing drug seeking during extinction and cued reinstatement of cocaine seeking, indicating that STN inactivation reduces economic demand for, and seeking of, cocaine (Bentzley and Aston-Jones, 2016). In addition, STN lesions also appear to reduce the motivation for alcohol and escalation of alcohol consumption under intermittent access or after withdrawal (Pelloux and Baunez, 2017). More recent work has also shown that STN DBS in rats prevents re-escalation of heroin consumption after abstinence (Wade *et al*, 2017) and prevents both the escalation of cocaine intake and re-escalation after prolonged, but not short, abstinence (Pelloux *et al*, 2018), highlighting the STN as a potential target for addiction therapeutics. However, other studies complicated this picture in finding that STN lesions enhance the primary reinforcing properties of cocaine during acquisition of low-dose cocaine self-administration, increase motivation for cocaine, and enhance both the induction and expression of cocaine sensitization (Uslaner *et al*, 2005). In addition, STN lesions appear to increase impulsive action in some behavioral tasks, but also decrease impulsive choice in others, with amphetamine and food restriction further amplifying this effect in STN-lesioned animals, suggesting STN lesions may exert complex behavioral effects through enhancement of the incentive salience of rewards

(Uslaner and Robinson, 2006). This idea was further explored in a study revealing that STN lesions increase “sign-tracking” conditioned responding to a conditioned light stimulus paired with food or cocaine rewards, indicating the STN may be involved in modulating the incentive salience of reward-related stimuli (Uslaner *et al*, 2008). During alcohol intake, STN lesions enhance the motivation for alcohol consumption and time spent in alcohol-paired environments only for alcohol-preferring “high-drinker” rats while having an opposite effect on motivation for alcohol and environmental preference in “low-drinker” rats, suggesting that not only reward-type, but also baseline reward preference, may influence how STN lesions impact motivated behavior (Lardeux and Baunez, 2008).

Taken together, this previous work presents a complex picture of how the STN influences motivated behavior, particularly in the context of rewarding drugs. One explanation for these disparate results may lie in the ability of incomplete lesions to induce functional and morphological reorganization in surviving brain tissue (Castro-Alamancos and Borrel, 1995). Partial lesions present additional complications for STN studies because human, nonhuman primate, and rat STN is thought to be topographically subdivided into motor, associative, and limbic segments loosely defined by their overlapping, but topographically biased, afferent connections (Alkemade, 2013; Haynes and Haber, 2013; Kita *et al*, 2014; Temel *et al*, 2005). While the functional territories suggested by these afferent connections are overlapping, the gradient heterogeneity in where STN afferents cluster nevertheless suggests that both the location and size of partial STN lesions could influence behavioral outcomes by disrupting some STN functions more than others, and similar localization problems may affect experiments utilizing pharmacological manipulation with microinfusion of drugs such as muscimol. It is

also possible that these contrasting results may be the product of manipulations differentially impacting specialized, segregated information streams within the STN that engage neuronal subpopulations responsive to separate reinforcers. There is indeed evidence that distinct groups of STN neurons may encode separate rewards (such as cocaine vs sucrose), along with reward magnitude and omission (Breysse *et al*, 2015; Lardeux *et al*, 2009; 2013). Finally, the specific patterning of STN activity, particularly neuronal beta band oscillations, may be a critical component of STN function, with maladaptive PD- (Brown *et al*, 2001; Eusebio *et al*, 2012; Limousin *et al*, 1995) and drug-related (Pelloux *et al*, 2018) behaviors occurring alongside abnormal oscillatory activity in the STN, which is disrupted by therapeutic STN DBS and lesions (Eusebio *et al*, 2012; Limousin *et al*, 1995; Pelloux *et al*, 2018). Such complications may highlight the need for studies that utilize reversible and projection-specific methods to dissect different functions of the STN, especially given that different STN afferents that may transmit distinct streams of information to subpopulations of STN neurons or differentially modulate the patterning of STN activity.

The corticosubthalamic hyperdirect pathway

While studies of direct STN manipulation may present a complex and incomplete picture of STN function, even less is known about the functional roles of specific STN afferents, especially corticosubthalamic hyperdirect pathway afferents. The hyperdirect pathway has frequently been left out of the traditional direct and indirect striatal pathway model, yet these projections have been known to exist for decades (Canteras *et al*, 1990; Nambu *et al*, 1996; Romansky *et al*, 1979). In fact, corticosubthalamic projections are diverse, originating from numerous cortical regions including the mPFC, anterior

cingulate, motor cortex, and somatosensory cortex, with primarily visual, auditory, and retrosplenial cortex being excluded from this pathway (Canteras *et al*, 1990). These hyperdirect projections were initially thought to temporally bound motor/behavioral programs between a short-latency hyperdirect and slower indirect pathway signal, which together would inhibit motor output before and after the selected time of an action (Nambu *et al*, 2002). This concept was expanded with later studies suggesting that the hyperdirect pathway provides a mechanism for top-down executive control of basal ganglia output by establishing a gating threshold for behavioral programs, particularly in situations of decision conflict (Cavanagh *et al*, 2011). This process likely involves hyperdirect projections from diverse cortical regions, but very little is known about the individual contributions from these cortical territories.

Although this concept has, for the most part, not been experimentally applied to addiction-related behavior, it seems likely that hyperdirect input plays some role, such as when negative outcomes of drug abuse or salient drug-associated cues create decision conflict during periods of drug use or abstinence respectively. As mentioned previously, dysregulation of the prefrontal cortex after chronic drug use is hypothesized to underlie a loss of inhibitory control generally observed among addicted individuals, and these changes are likely a key factor in drug relapse (Goldstein and Volkow, 2011; Kalivas, 2009). Recent work has highlighted the importance and distinct contributions of OFC, anterior cingulate cortex, prelimbic cortex, and infralimbic cortex in addiction-related behaviors (Garcia *et al*, 2018; Goldstein and Volkow, 2011; Hogarth *et al*, 2013; Kalivas, 2009; Lucantonio *et al*, 2012; 2014; Peters *et al*, 2013; Rogers *et al*, 2008; Schoenbaum and Shaham, 2008; Van den Oever *et al*, 2010; 2013; Volkow and Morales, 2015; Yager

et al, 2015), and while all of these regions send hyperdirect projections to STN (Haynes and Haber, 2013; Maurice *et al*, 1998), few of these projections have been studied in the context of motivated or addiction-related behavior. Contralateral disconnection of the mPFC and STN during a test of visual attention has been shown to reduce discriminative accuracy, increase perseveration, and slow response latencies in contrast to animals with combined ipsilateral mPFC and STN lesions, suggesting that corticosubthalamic projections help regulate attention and perseveration (Chudasama *et al*, 2003). In addition, contralateral disconnection experiments suggest that prelimbic cortex and STN may together utilize cue information to facilitate behavioral switching when environmental contingencies change (Baker and Ragozzino, 2014), but that medial STN may not be involved in medial OFC regulation of cocaine self-administration maintenance (Kantak *et al*, 2013). Specific prelimbic cortex, infralimbic cortex, and anterior cingulate cortex hyperdirect projections have yet to be closely examined in the context of addiction-related behavior.

This limited number of studies may suggest that hyperdirect projections originating in prelimbic cortex represent the most promising target for manipulation during addiction-related behavior given their involvement in behavioral switching. In addition, prelimbic cortex is already broadly implicated in these behaviors, with photoinhibition of prelimbic cortex enhancing cocaine self-administration (Martín-García *et al*, 2014) while also reducing cocaine-primed reinstatement (Martín-García *et al*, 2014; Stefanik *et al*, 2013). This potential for involvement of prelimbic cortex hyperdirect projections in addiction-related behavior is explored later in this work (Chapter 3).

1.5 Thesis Overview

The overall aim of the work described in this thesis is to utilize virally-mediated chemogenetic techniques in parallel with animal models of addiction to better dissect and understand the function of corticostriatal and subthalamic circuits in addiction-related behavior, including subthalamic afferents along the indirect and hyperdirect pathways. This is achieved through expression of $G_{i/o}$- and G_q-coupled DREADDs to bidirectionally manipulate the activity of both brain nuclei and discrete neuronal projections. Chapter 2 describes an investigation of how corticostriatal afferents between the medial prefrontal cortex and nucleus accumbens modulate responses to psychostimulants and drug-paired cues using locomotor sensitization to amphetamine and cocaine self-administration as models of addiction-related behavior. Chapter 3 examines the role of the subthalamic nucleus in locomotor sensitization to amphetamine through bidirectional manipulation of subthalamic nucleus activity, along with targeted inhibition of indirect pathway inputs from the ventral pallidum and hyperdirect pathway inputs from prelimbic cortex. Chapter 4 provides a summary and discussion of this work along with potential future directions.

Chapter 2

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

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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 before 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 that 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 and Volkow, 2011; Moussawi *et al*, 2011; Shiflett and Balleine, 2011). 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 and Hyman, 2000; Lüscher and Malenka, 2011; Nestler, 2001; Russo *et al*, 2010; Schmidt and Pierce, 2010; Steketee and 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 and Volkow, 2011; Kalivas and Volkow, 2005; Steketee and 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 and Faull, 1989; Wall *et al*, 2013), and hence 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 and 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, resulting in membrane hyperpolarization and inhibition of neuronal firing (Armbruster *et al*, 2007; Ferguson *et al*, 2011; Sternson and Roth, 2014 for review). 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 affects 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-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 promotor and expressing $G_{i/o}$-DREADDs (AAV-hSyn-DIO-hM_4Di-mCherry; hM_4Di) 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 ~1 × 109 viral genomes/µl). 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 1× Hanks' balanced saline solution at a titer of ~2.5 × 109 viral genomes/µl as described previously (Kremer et al, 2000).

Experiment 1 (c-Fos)

Male Long-Evans rats (*n* = 22, Charles River) weighing ~250-300 g 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% isoflurane (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 were A/P 3.2, M/L ±1.4, and D/V -3.5 from skull surface, and for NAc were A/P 1.7, M/L ±1.0, and DV -6.5. For assessment of cFos in the NAc, 2 µl of GFP was injected in one hemisphere of the mPFC, 2 µl of hM4Di was infused into the contralateral hemisphere of the mPFC, and 2 µl of CAV-Cre was infused bilaterally into the NAc over a 10-min period at a flow rate of 0.2 µl/min. Thus, each rat (*n* = 7) had GFP in one hemisphere and hM4Di in the other hemisphere, allowing 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 hM_4Di (*n* = 7) in the mPFC, allowing for a between-subject design. Accuracy of injection coordinates was confirmed by visualization of GFP or mCherry immunofluorescence in mPFC. At 20 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*). After 2 h, rats were killed 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 were A/P 2.8, M/L ±0.8, and D/V −4.5 from skull surface, and for NAc were A/P 1.8, M/L ± 1.0, and DV -7.5, and rats received 1 µl 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). At 20 min before 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 min. Then they received an injection of saline followed by 30 min 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 and 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: hM_4Di 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 hM_4Di (*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 (20mg/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 µl of 0.9% sterile saline administered over 4 sec; obtained from the National Institute on Drug Abuse) during their light cycle on a fixed interval 20 sec (FI:20) schedule of reinforcement. Each cocaine infusion was paired with a 5 sec light and tone stimulus. Self-administration sessions lasted 2 h 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 and 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 because of 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 before PR testing for three sessions to obtain a baseline response. Rats were then administered CNO (3 mg/kg, *ip*) 20 min before PR testing for three sessions. Following testing, rats underwent three additional PR sessions in the absence of any pretreatment to determine whether CNO treatment had any lasting effects on PR responding.

A subset of rats (GFP: $n = 7$; hM_4Di: $n = 5$) 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 before 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 before the cocaine injections. Cocaine injections were given immediately before reinstatement testing, and levers were extended for these sessions but responding did not have any programmed consequences. These pretreatments were given in a counterbalanced manner and rats received additional extinction training sessions following the first reinstatement test (see Figure 4 for illustration of experimental design). In all, 13 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 (42 SD away from the mean).

Immunohistochemistry

Rats were anesthetized with Beuthanasia-D (Schering- Plough) and perfused transcardially with 1× PBS (pH 7.4), followed by 4% paraformaldehyde (PFA). Brains were extracted, post-fixed in 4% PFA overnight, and stored in 1× PBS. Floating sections (40-60 μm) 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 coverslipped with Vectashield mounting medium with DAPI (Vectorlabs). Z-stack images were captured with a Zeiss confocal microscope and compressed into a single plane before 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 are graphed as mean ± 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 hM_4Di receptors in mPFC neurons projecting to the NAc in order to selectively and transiently decrease activity of these afferents (Figure 2.1a and b). Viral expression in the mPFC was largely confined to the cingulate and prelimbic regions (Figure 2.1). 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, the two output regions of the mPFC that also send glutamatergic inputs into the NAc. We observed a strong amount of fluorescence in the NAc (Figure 2.1c), with some fluorescence in the BLA (Figure 2.1d) and no fluorescence in the VH (Figure 2.1e), suggesting that DREADD receptor expression was occurring primarily in mPFC neurons that were projecting selectively to the NAc.

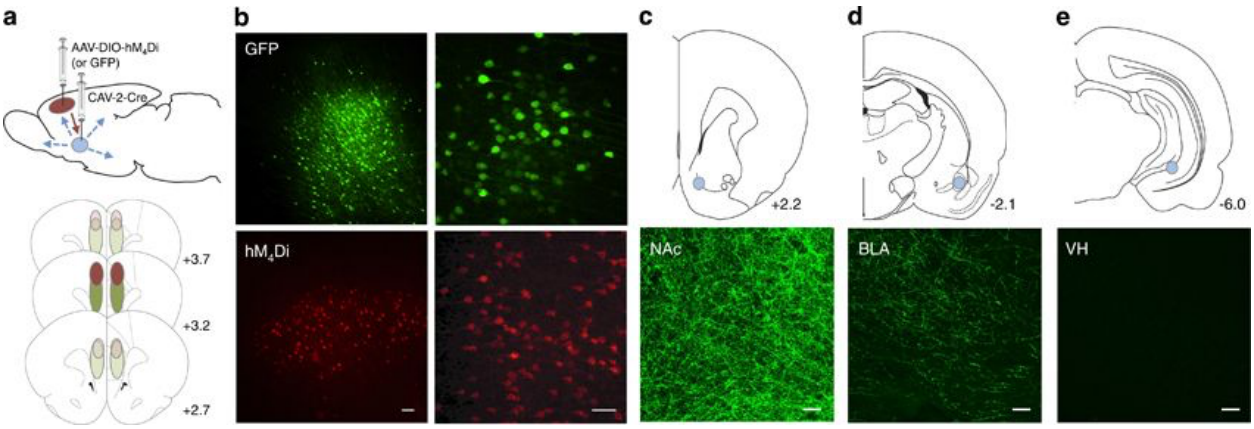

Figure 2.1. Viral vector expression. (**a**, top) Illustration of the intersectional viral vector approach: Cre-recombinase (Cre)-dependent AAV vectors (hM$_4$Di or GFP) were injected into the medial prefrontal cortex (mPFC) and the retrograde CAV-Cre virus was injected into the nucleus accumbens (NAc). (**a**, 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 hM$_4$Di (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.

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 hM$_4$Di 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, 2011, see Sternson and Roth, 2014 for review). This effect has been observed in glutamatergic pyramidal neurons of the cortex, the population of neurons that expressed hM$_4$Di receptors in the present set of experiments (Katzel 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 studies, activation of hM$_4$Di receptors in mPFC significantly decreased the number of cocaine-evoked c-Fos+ cells in mPFC by ~30% compared with the GFP controls (Figure 2.2a and b; $t_{14} = 2.68$, $p = 0.02$). In order

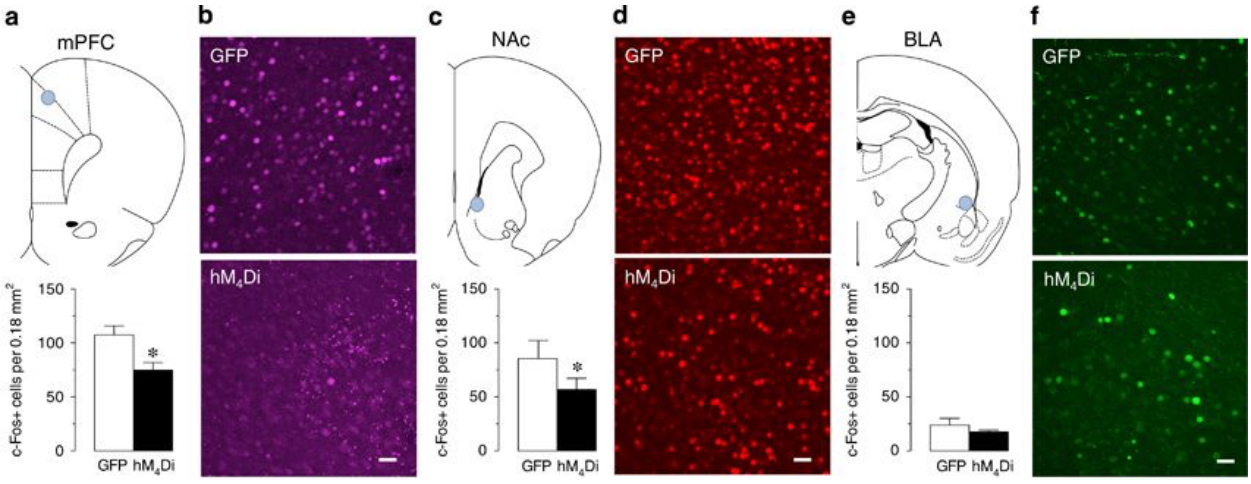

Figure 2.2. c-Fos expression. (**a**, **c**, and **e**) CNO-mediated activation of hM$_4$Di-expressing mPFC neurons significantly reduces the number of cocaine-induced c-Fos+ cells in PFC (**a**, *$p < 0.05$ *versus* GFP group, $n = 6$-10) and NAc (**c**, *$p < 0.05$ vs GFP hemisphere, $n = 7$) but not in BLA (**e**, $p = 0.38$ versus GFP group, $n = 7$-8). (**b**, **d**, and **f**) Representative sections of Fos immunohistochemistry in mPFC (**b**), NAc (**d**), and BLA (**f**) are shown from GFP (top) and hM$_4$Di sections (bottom). Scale bars, 40 μm.

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 hM$_4$Di receptors in mPFC significantly decreased the number of cocaine-evoked c-Fos+ cells in NAc by ~32% compared with the GFP control hemisphere (Figure 2.2c and d; $t_6 = 0.56$, $p = 0.01$), suggesting that decreasing activity of mPFC projections

to NAc reduces the neuronal activity of its downstream targets. In contrast, CNO-induced activation of hM_4Di receptors in mPFC had no effect on cocaine-evoked c-Fos+ cells in the BLA (Figure 2.2e and 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 hM_4Di receptors had no effect on locomotor activity following saline injections (Figure 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 hM_4Di rats (Figure 2.3a and 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, $F_{3,72} = 0.84$, $p = 0.48$), hM_4Di rats had significantly reduced levels of locomotor activity during the last session compared with GFP controls ($p < 0.05$), suggesting that dampening activity of mPFC afferents to NAc attenuates the induction of locomotor sensitization.

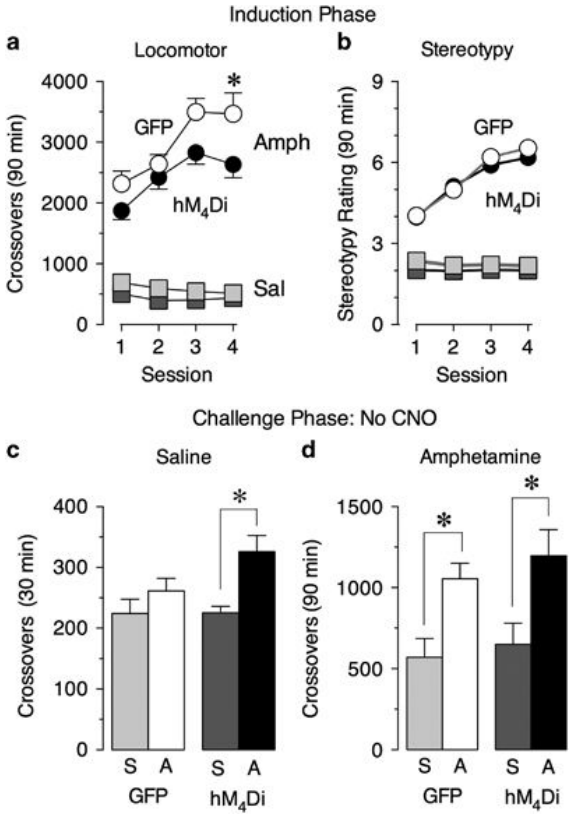

Figure 2.3. Amphetamine sensitization. Decreasing activity of mPFC afferents to NAc alters the development of amphetamine sensitization. CNO-mediated activation of hM_4Di in corticostriatal afferents significantly decreased locomotor sensitization to amphetamine as measured by crossovers (**a**, $*p < 0.05$ *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 (**c**, $*p < 0.05$ vs hM_4Di 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**, $*p < 0.05$ vs respective control groups given saline during the induction phase). Data represent mean ±SEM. Black symbols/shading (hM_4Di) and white symbols/shading (GFP) represent groups that received amphetamine treatment during the induction phase. Dark gray symbols/shading (hM_4Di) and light gray 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.

Following 2 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 min 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, hM_4Di rats that had previously received amphetamine showed enhanced locomotor activity during this test compared with the hM_4Di rats that had received saline, an effect not observed in the GFP groups (Figure 2.3c; main effect of drug pretreatment $F_{1, 46} = 2.25$, $p = 0.002$), suggesting that only hM_4Di 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 hM_4Di 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 (Figure 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 (Figure 2.4). During the last three PR training sessions before testing, both GFP and hM_4Di rats made significantly more active lever responses

compared with inactive lever responses (Figure 2.4a; main effect of lever, hM$_4$Di: $F_{1, 28}$ = 51.70, $p < 0.0001$; GFP: $F_{1,12}$ = 50.73, $p < 0.0001$) but there were no differences in lever responding across sessions (Figure 2.4a; no main effect of session, hM$_4$Di: $F_{2, 56}$ = 1.57, p = 0.38; GFP: $F_{2, 24}$ = 0.37, p = 0.70; no interaction between lever and session, hM$_4$Di: $F_{2, 56}$ = 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 (Figure 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 (Figure 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 hM$_4$Di 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). There were no differences between groups in active lever responses (Figure 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 (Figure 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.

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	7-10	1	1	3	1

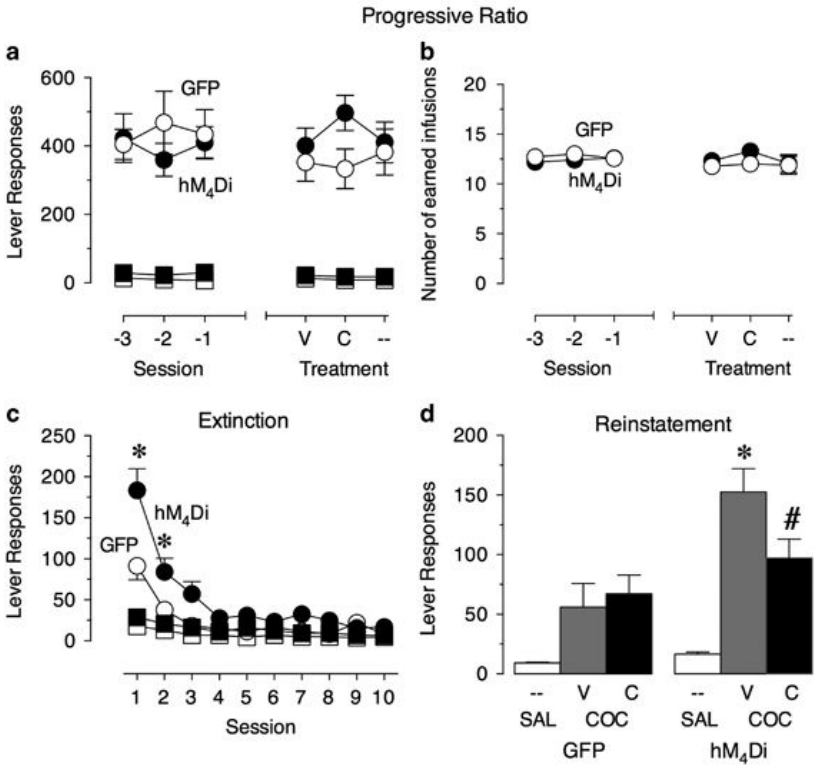

Figure 2.4. Cocaine self-administration. Decreasing activity in mPFC afferents to NAc modulates drug seeking. There were no differences between groups in active (circles) and inactive (squares) lever responses (**a**, left panel) and number of earned infusions (**b**, left panel) during baseline progressive ratio (PR) cocaine self-administration (last 3 sessions before testing). CNO-mediated activation of hM_4Di in corticostriatal afferents had no effect on lever responses (**a**, right panel) or number of earned infusions (**b**, right panel). Rats that had hM_4Di activation during PR showed a significant increase in the number of active lever responses during the first 2 sessions of extinction training **(c**, $^{*}p < 0.05$ vs GFP group) as well as following a cocaine prime (**d**, $^{*}p < 0.05$ vs GFP group). CNO pretreatment before a cocaine prime significantly attenuated active lever responding only in the hM_4Di group (d, $^{\#}p < 0.05$ vs V-treated hM_4Di group). Data represent mean ±SEM. Black symbols in line graphs represent the hM_4Di group and white symbols represent the GFP controls. [X]Note 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.

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, hM$_4$Di rats that had received CNO treatment during PR testing made significantly more active lever responses during the first 2 days of extinction training compared with the GFP controls (Figure 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, hM$_4$Di rats made significantly more active lever responses following a priming injection of cocaine compared with the GFP controls (Figure 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 before the cocaine prime significantly attenuated reinstatement in the hM$_4$Di rats (Figure 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 (Figure 2.4c; no main effect of virus $F_{1, 10} = 3.70$, $p = 0.08$; 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 (Figure 2.4d; $p = 0.80$).

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 hM_4Di receptor activation, which is to reduce excitability of the cells expressing the $G_{i/o}$-DREADDs (Armbruster et al, 2007; Ferguson et al, 2011, see Sternson and Roth, 2014 for review), 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 because of axons passing through the region. In line with this idea, we found that activation of hM_4Di 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 and Garcia, 1998); thus, it is likely that the reduction in c-Fos that we observed following hM_4Di receptor activation of corticostriatal neurons was because of 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, 2004; Steketee and Kalivas, 2011; Tzschentke and Schmidt, 2003; Vanderschuren and 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 and 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 that are thought to be regulated by the dorsal striatum (Staton and Solomon, 1984). Unexpectedly, locomotor sensitization appeared equivalent between groups during the challenge phase. There are several possible explanations that could account for these results. First, 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. Second, 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. Third, 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 $G_{i/o}$-DREADDs not only attenuated the development of cocaine sensitization but also blocked cocaine-induced increases in the frequency of miniature excitatory postsynaptic 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 and Schmidt, 2003).

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 before 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 and Kalivas, 2013; Stefanik *et al*, 2016). In addition, it is consistent with recent work demonstrating that reversing silent synapse remodeling (that 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 and 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 with 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 that 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 and 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 and Robbins, 2005; LaLumiere *et al*, 2012; Marchant *et al*, 2014), 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 and 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 and Lefkowitz, 2006; Wettschureck and Offermanns, 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 noteworthy 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 (that 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 and 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 and 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

DREADD-mediated modulation of the subthalamic nucleus and its afferents in locomotor sensitization to amphetamine

*This chapter is currently in preparation as an article for publication with Ellen Yin, Elissa Sutlief, and Susan M. Ferguson as co-authors.

KGN, EY, and ES performed the behavioral and immunohistochemical experiments. KGN and SMF designed the experiments and wrote the manuscript.

3.1 Abstract

Preclinical studies suggest the subthalamic nucleus (STN) plays a critical role in behaviors related to addiction and that therapies which manipulate STN activity (such as deep brain stimulation) may eventually show promise for treating substance use disorders. However, most studies have directly manipulated the STN through lesioning, deep brain stimulation, and pharmacological microinfusion, resulting in complex behavioral results and overlooking the roles of distinct STN afferents. To address this, we used viral vectors to express DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) in the STN of rats and bidirectionally manipulate STN activity during the induction of amphetamine sensitization, while in separate experiments utilizing a Cre-recombinase dependent $G_{i/o}$-coupled DREADD to inhibit indirect pathway afferents from ventral pallidum and hyperdirect pathway afferents from prelimbic cortex. Stimulation of STN neurons with G_q-DREADDs had no effect on the acute locomotor response to amphetamine; however, this manipulation completely blocked the development and persistence of locomotor sensitization. In addition, STN stimulation induced mild hyperactivity in amphetamine-free DREADD controls. In contrast, inhibition of STN neurons with $G_{i/o}$-DREADDs enhanced the induction of amphetamine sensitization without altering conditioned responding to injection procedures or persistence of sensitization. In addition, inhibition of STN neurons had no effect on the acute locomotor response to amphetamine, nor did it alter locomotor activity in amphetamine-free controls. Inhibition of afferents from ventral pallidum had no effect on the induction or persistence of amphetamine sensitization but blocked conditioned responding. Surprisingly, inhibition of afferents from prelimbic cortex had no effect on induction of sensitization but attenuated

both conditioned responding and persistence of sensitization. These results suggest the STN and its afferents play more complex roles in the regulation of addiction-like behavior than previously recognized and stress the need for both further characterization of STN afferents and integration of inputs within STN.

3.2 Introduction

The corticolimbic circuitry involved in addiction-related behavior has been extensively studied, and psychostimulant drugs in particular are known to initiate a broad range of intra- and extracellular adaptations believed to underlie the transition from controlled drug use to compulsive drug abuse (Baik, 2013; Berke and Hyman, 2000; Berridge and Arnsten, 2013; Everitt *et al*, 2008; Lobo and Nestler, 2011; Lüscher and Malenka, 2011; Nestler, 2001; van Huijstee and Mansvelder, 2014; Yager *et al*, 2015). A common experimental approach for probing these adaptations is induction of locomotor sensitization to drugs, which serves as a behavioral proxy for drug-induced plasticity through analysis of the enhanced psychomotor activating and incentive motivational effects of drugs exerted in part by changes to dopaminergic signaling following repeated drug exposure. These signaling changes lead to lasting sensitization of neural circuits thought to underlie salience attribution and contribute to the long-term behavioral consequences of addiction (Paulson and Robinson, 1991; Robinson and Becker, 1986; Robinson and Berridge, 2008; Segal and Mandell, 1974; Wise and Bozarth, 1987).

While this and other models of addiction have long been used to study the function of corticolimbic addiction circuitry, relatively few studies have focused on local circuitry of the subthalamic nucleus (STN), which only recently rose to prominence as a potential therapeutic target for treating addiction (Baunez *et al*, 2005; Bentzley and Aston-Jones, 2016; Luigjes *et al*, 2012; Pelloux and Baunez, 2013; 2017; Pelloux *et al*, 2018; Pierce and Vassoler, 2013; Uslaner *et al*, 2005). Previous studies show that disruption of STN activity with lesions (Baunez *et al*, 2005; Pelloux and Baunez, 2017), microinfused GABA agonists (Bentzley and Aston-Jones, 2016), and deep brain stimulation (DBS) (Pelloux *et*

al, 2018; Rouaud *et al*, 2010; Wade *et al*, 2017) can reduce motivation for addictive drugs, escalation of use, and/or reinstatement of drug seeking. However, other studies show STN lesions may increase motivation for cocaine (Uslaner *et al*, 2005), enhance locomotor sensitization to cocaine (Uslaner *et al*, 2005), enhance the incentive salience of rewards (Uslaner *et al*, 2005; 2008), and increase both motivation for alcohol and alcohol conditioned-place-preference (CPP) in alcohol-preferring rats (Lardeux and Baunez, 2008), sometimes in clear contradiction with similar work. Taken together, this evidence presents a complex and contradictory picture of how the STN might influence addiction-related behavior.

A number of possibilities may explain these conflicting results including compensatory reorganization around partial brain lesions (Castro-Alamancos and Borrel, 1995), inconsistent targeting of STN neuronal subpopulations with variable reward response properties (Breysse *et al*, 2015; Lardeux *et al*, 2009; 2013), and differential modulation of patterned STN activity, including neuronal beta band oscillations implicated in disordered behavior (Brown *et al*, 2001; Eusebio *et al*, 2012; Limousin *et al*, 1995; Pelloux *et al*, 2018). Another possibility involves irregular targeting, or engagement by DBS, of neuronal subpopulations with distinct afferent or efferent connections that both partially integrate and partially segregate separate streams of information. Human, nonhuman primate, and rat STN are all thought to be partially subdivided into motor, associative, and limbic segments defined by their afferent connections (Alkemade, 2013; Haynes and Haber, 2013; Kita *et al*, 2014; Temel *et al*, 2005), yet the contributions of discrete projections to and from STN remain poorly characterized. Previous work suggests neurons in ventral pallidum that project to STN along the indirect pathway are

recruited during context-induced reinstatement of extinguished alcohol seeking, with chemogenetic disconnection of ventral pallidum and STN reducing reinstatement and reacquisition of alcohol seeking, along with motivation for alcohol under a progressive ratio test (Prasad and McNally, 2016). The role of corticosubthalamic (hyperdirect pathway) afferents in addiction-related behavior also remains unclear, though medial STN afferents from orbitofrontal cortex show no evidence of regulating cocaine self-administration (Kantak *et al*, 2013), while afferents originating in prelimbic cortex may regulate attention and perseveration (Chudasama *et al*, 2003), while also facilitating behavioral switching when environmental contingencies change (Baker and Ragozzino, 2014). To date, no other studies have examined STN afferents under models of addiction behavior, and the role of STN afferents in locomotor sensitization remains unknown.

To address these questions, we first used viral vectors to express DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) in the STN of rats during induction of amphetamine sensitization, allowing reversible modulation of STN activity with excitatory G_q-coupled and inhibitory $G_{i/o}$-coupled DREADDs previously demonstrated to excite or inhibit neuronal signaling through changes in cAMP signaling, stimulated c-Fos expression, and action potential firing (Armbruster *et al*, 2007; Burnett and Krashes, 2016; Ferguson and Neumaier, 2012; Ferguson *et al*, 2011; 2013; Kerstetter *et al*, n.d.; Roth, 2016; Smith *et al*, 2016; Wunsch *et al*, 2017; Yager *et al*, 2019). We next inhibited STN afferents originating in either ventral pallidum or prelimbic cortex during induction of amphetamine sensitization using a Cre-recombinase dependent $G_{i/o}$-coupled DREADD approach. To control for the possibility of off-target behavioral effects from our DREADD ligand clozapine N-oxide (CNO) (Gomez *et al*, 2017;

MacLaren *et al*, 2016; Manvich *et al*, 2018), we also tested the effects of CNO on DREADD-free rats during induction of amphetamine sensitization. Altogether, this approach was used to compare how manipulation of STN and two of its critical afferents alters the induction and persistence of drug sensitization, providing new evidence for how STN circuitry might regulate this behavior.

3.3 Materials and Methods

Experimental Strategy

The overall experimental strategy for characterizing the role of the STN and its afferents in amphetamine sensitization was to bi-directionally modulate the STN utilizing viral chemogenetics, as well as to use a separate combinatorial viral chemogenetic approach selectively modulating afferents originating in both the ventral pallidum (via the indirect pathway) and prelimbic cortex (via the hyperdirect pathway), during the induction of locomotor sensitization to amphetamine. In experiment 1, amphetamine sensitization was induced in control animals without any viral modification or DREADD expression to test for off-target behavioral effects resulting from the CNO administration used to activate DREADD receptors. In experiments 2 and 3, the effects of stimulating STN activity with activation of G_q-DREADDs and dampening activity with activation of $G_{i/o}$-DREADDs were examined during the induction of amphetamine sensitization, and lasting effects on conditioned responding to the drug-associated injection procedure and to the persistence of sensitization were later examined in the absence of further DREADD activation. In experiments 4 and 5, afferent inputs to the STN from the ventral pallidum and prelimbic cortex were inhibited during induction of amphetamine sensitization following bilateral

injection of $G_{i/o}$-DREADD-expressing Cre-dependent adeno-associated virus (AAV) into either the ventral pallidum or prelimbic cortex paired with bilateral injection of a retrogradely transported Cre-expressing canine adenovirus (CAV) into the STN. Lasting effects of prior DREADD activation on conditioned responding and persistence of sensitization were again examined in the absence of additional DREADD activation. All experiments were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and followed National Institutes of Health (NIH) guidelines.

Animal use

Male Sprague Dawley rats (Envigo) weighing 250-274g were acclimatized upon arrival to the housing environment for at least 3 days prior to any experimental manipulation. All animals were pair housed for the duration of the study and maintained on a 12:12 h light-dark cycle in a temperature- and humidity-controlled vivarium with *ad libitum* access to food and water.

Drugs

Clozapine-*N*-oxide (CNO) was obtained from the NIH as part of the Rapid Access to Investigate Drug Program funded by the NINDS. CNO was administered into the intraperitoneal cavity (*ip*) in a volume of 1 mL/kg at a dose of 5 mg/kg. CNO was dissolved in 100% DMSO, then diluted in sterile water for a final concentration of 6% DMSO. Vehicle injections consisted of 6% DMSO in sterile water. Amphetamine (Sigma) was dissolved in sterile 0.9% saline and administered *ip* in a volume of 1 mL/kg at a dose of either 1.5 mg/kg (amphetamine sensitization) or 0.5 mg/kg (amphetamine challenge). Meloxicam (1

mg/kg *sc*) and Beuthanasia-D (1 mL of 50% concentration, *ip*) were obtained from Patterson Veterinary and dissolved in 0.9% sterile saline as appropriate.

Viral Vectors

Non-Cre dependent G_q- and $G_{i/o}$-DREADD constructs driven by the human synapsin promotor and packaged in AAV serotype 5 (AAV5-hSyn-hM_3D_q-mCherry and AAV5-hSyn-hM_4D_i-mCherry) were developed by Dr. Bryan Roth and obtained from the University of North Carolina viral vector core (titer of ~1×10^9 viral genomes/µl). A Cre-dependent $G_{i/o}$-DREADD construct (AAV8-hSyn-DIO-hM_4D_i-mCherry) also developed by Bryan Roth (University of North Carolina) was packaged in AAV serotype 8 by, and obtained from, Addgene (titer of ~1×10^9 viral genomes/µl). A Cre-recombinase-expressing canine adenovirus (CAV2-Cre) previously developed by Dr. Eric Kremer was prepared in dog kidney (DK/E1-1) cells, purified by sucrose and CsCl gradient centrifugation, and re-suspended in 1× Hanks' balanced saline solution at an approximate titer of 2.5×10^9 viral genomes/µl as previously described (Kremer et al., 2000) and obtained from Dr. John Neumaier (University of Washington).

Surgical Techniques

During all surgical procedures, rats were anesthetized with inhaled isoflurane (4% induction, 2% maintenance) and administered meloxicam prior to surgery for analgesia. Using standard stereotaxic procedures, 33-gauge needles attached to gas-tight Hamilton syringes 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, anterior/posterior, medial/lateral, dorsal/ventral, injection volume in µL): prelimbic cortex, +3.2, ±0.8, -3.5, 0.6; subthalamic nucleus (STN), -3.8, ±2.4, -8.35, 0.3; ventral pallidum,

-0.1, ±2.4, -8.2, 0.5. All viruses were bilaterally infused in the region of interest at a rate of 300 nL/min, and needles were left in place for an additional 5 minutes to allow for diffusion away from the injection site. For STN stimulation, AAV5-hSyn-hM_3D_q-mCherry was injected into the STN of all rats. For STN inhibition, AAV5-hSyn-hM_4D_i-mCherry was injected into the STN. When inhibiting STN afferents, retrogradely transported CAV2-Cre was injected into the STN and the complementary Cre-dependent AAV8-hSyn-DIO-hM_4D_i-mCherry virus was injected into the ventral pallidum (experiment 4) or prelimbic cortex (experiment 5) such that only neurons projecting to STN from either the ventral pallidum or prelimbic cortex would receive both viral vectors and express Cre-dependent $G_{i/o}$-DREADDs. All animals had at least 3 days of post-operative recovery and monitoring following stereotaxic infusions.

Locomotor Sensitization

The psychomotor activating effects of amphetamine were measured using locomotor activity boxes (San Diego Instruments), and ambulations were recorded as two consecutive infrared beam breaks. At least fourteen days following stereotaxic surgery, rats (*n* = 138) received either 8 (experiments 2 and 3) or 6 (experiments 4 and 5) injections of either amphetamine (1.5 mg/kg, *ip*) or saline over 11-15 days (one injection every other day). Fifty minutes prior to each injection, rats received a 30 min habituation to the locomotion chambers followed by an injection of either CNO (5 mg/kg, *ip*) or vehicle (6% DMSO in sterile water, *ip*) and another 20 min habituation for pretreatment to take effect. Following amphetamine or saline injection, rats were placed back into the locomotor activity boxes and ambulations were recorded for 90 min. Rats were also observed during all sensitization sessions by an experimenter blind to the treatment conditions and

assigned a locomotor-stereotypy rating during each observation (30 sec observation each every 5 min for the 90 min test session) using an adapted 9-point stereotypy rating scale (1 = asleep; 2 = inactive; 3 = normal in-place activity; 4 = normal, alert, active ambulation; 5 = hyperactive; 6 = slow-patterned stereotyped behaviors; 7 = fast-patterned stereotyped behaviors; 8 = restricted stereotyped behaviors; 9 = dyskinetic-reactive) (Dougherty and Ellinwood, 1983). After a 15-day withdrawal period, all rats underwent a challenge session where they received a 30 min habituation to the locomotor activity chambers followed by an *ip* injection of saline and additional 30 min of locomotor testing to detect a conditioned response to the drug-associated injection procedures. All rats then 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 the persistence of sensitization (see Figure 3.1 for full sensitization timeline). For experiment 1, an additional 38 rats without prior viral manipulation underwent this same procedure with 8 sessions of sensitization induction over 15 days. 28 of those rats were removed prior to the challenge session for a separate study, and the remaining 10 continued with 15 days of withdrawal and a challenge session to assess conditioned responding and persistence of sensitization. In all, 12 rats were excluded from analysis because of a lack of bilateral viral expression, 2 from the challenge session of experiment 3 due to missed *ip* injections, and an additional 5 confirmed to lack any viral expression by immunohistochemical analysis were included for data analysis in experiment 1 with rats lacking viral manipulation. Specifically, these 5 additional rats were confirmed to lack viral expression by two separate immunohistochemical analyses of the STN, where needle tracks were confirmed, and surrounding brain regions using brain slices extending from +2 to -6 mm from Bregma.

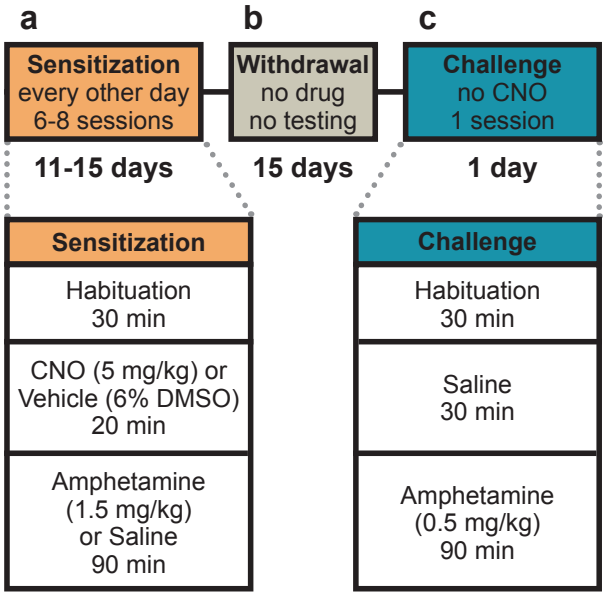

Figure 3.1. Amphetamine sensitization timeline and details. (**a**) At least 14 days after stereotaxic viral infusions, rats were sensitized to amphetamine. Sessions began with a 30 min habituation to locomotor activity boxes followed by pretreatment with either CNO (5 mg/kg) or vehicle (6% DMSO in sterile water) *ip* injection and an additional 20 min of habituation for pretreatment to take effect before *ip* injection of either amphetamine (1.5 mg/kg) or saline and placement of rats back in locomotor activity boxes for 90 min. Sensitization sessions were every other day for either 6 or 8 sessions over 11 or 15 days respectively. (**b**) After the final sensitization session, rats underwent drug withdrawal without locomotor testing in home cages for 15 days. (**c**) After withdrawal, all rats underwent a single challenge session consisting of a 30 min habituation, *ip* injection of saline, 30 min locomotor test for detection a conditioned response to drug-associated injection procedures, *ip* injection of a low-dose amphetamine challenge (0.5 mg/kg), and 90 min locomotor test to assess persistence of amphetamine sensitization.

Immunohistochemistry

Rats were anesthetized with Beuthanasia-D and perfused transcardially with 1× PBS (pH 7.4), followed by 4% paraformaldehyde (PFA). Brains were extracted, post-fixed in 4% PFA overnight, and then transferred to 1× PBS. Brains were sectioned in 40 µm slices using a Leica vibrating microtome. Floating sections were then washed in 0.5% Triton-X/PBS for 10 min, blocked in 5% normal goat serum (NS) in 0.25% Triton-X/PBS) for 2 h, and incubated in 2.5% NS-0.25%Triton-X/PBS containing antibodies against

dsred (1:400, rabbit host, Takara Bio Clontech, #632496) with gentle agitation on a standard analog shaker (VWR) overnight at room temperature. Sections were then rinsed 4 times in PBS and incubated in 2.5% NS/PBS containing goat anti-rabbit Alexa568 conjugated secondary antibody (1:250, Invitrogen, A-11036) for 2 h at room temperature with gentle agitation. They were then rinsed 2 times in PBS for 10 min, mounted on slides, and cover-slipped with Vectashield mounting medium with DAPI (Vector Labs, H-1500). Z-stack images were captured using a Zeiss LSM 710 confocal microscope and compressed into a single plane using ImageJ software (NIH).

Data Analysis

All statistical analyses were determined prior to running experiments and carried out with GraphPad Prism 7. Group differences in ambulations during sensitization induction were analyzed using repeated measures (RM) two-way ANOVA for session × pretreatment (CNO vs vehicle) as well as session × amphetamine treatment (amphetamine vs saline) followed by Bonferroni's post hoc tests when appropriate. For the challenge session, ambulations were analyzed using a two-way ANOVA of CNO history (CNO vs vehicle during sensitization induction) × amphetamine history (amphetamine vs saline during sensitization induction) followed by Bonferroni's post hoc tests when appropriate. For all comparisons, $\alpha \leq 0.05$. Data are graphed as mean ± SEM.

3.4 Results

Expression of STN targeted DREADDs is most concentrated in central and medial STN, and the intersectional viral vector approach effectively limits expression to STN-projecting prelimbic and ventral pallidum neurons

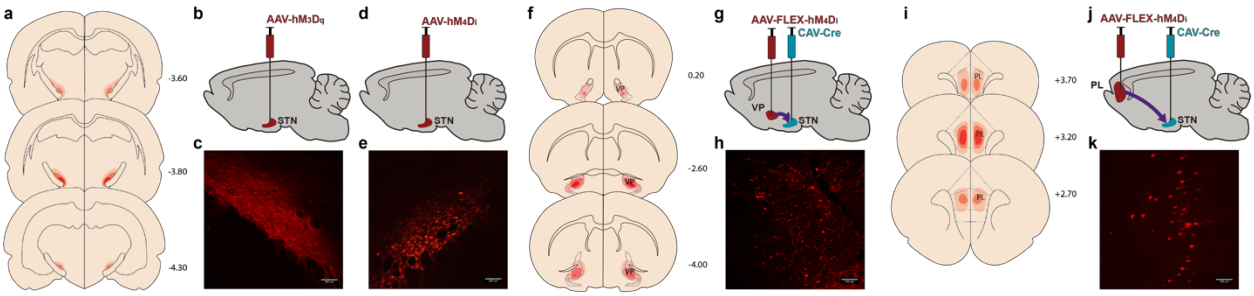

Figure 3.2. Viral expression of DREADD receptors. Scale bars represent 100 µm. **(a)** Distribution of G_q- and $G_{i/o}$-DREADD expression in the STN for experiments 2 and 3. Light pink represents the furthest extent of viral expression spread observed in experimental animals, dark pink represents the average spread of expression, and red represents expression observed in all experimental animals. (**b**) Strategy for expressing G_q-DREADDs in the STN. (**c**) Representative image of G_q-DREADD expression in the STN. (**d**) Strategy for expressing $G_{i/o}$-DREADDs in the STN. (**e**) Representative image of $G_{i/o}$-DREADD expression in the STN. **(f)** Distribution of $G_{i/o}$-DREADD expression in the ventral pallidum for experiment 4. Light pink represents the furthest extent of viral expression spread observed in experimental animals, dark pink represents the average spread of expression, and red represents expression observed in all experimental animals. (**g**) Strategy for expressing Cre-dependent $G_{i/o}$-DREADDs in VP-STN indirect pathway afferents. (**h**) Representative image of $G_{i/o}$-DREADD expression in the ventral pallidum. **(i)** Distribution of $G_{i/o}$-DREADD expression in the prelimbic cortex for experiment 5. Light pink represents the furthest extent of viral expression spread observed in experimental animals, dark pink represents the average spread of expression, and red represents expression observed in all experimental animals. (**j**) Strategy for expressing Cre-dependent $G_{i/o}$-DREADDs in prelimbic-STN hyperdirect pathway afferents. (**k**) Representative image of $G_{i/o}$-DREADD expression in the prelimbic cortex.

In experiments 2 and 3, viral vectors were used to express G_q- and $G_{i/o}$-DREADDs directly in STN neurons (Figure 3.2b and d). Immunohistochemical analysis revealed that expression of STN targeted DREADDs was most commonly concentrated in central and medial STN, with only a minority of animals showing limited expression in nearby ventral zona incerta, lateral hypothalamus, or parasubthalamic nucleus (Figure 3.2a, c, and e). In experiment 4, a Cre-dependent intersectional vector approach was used to express $G_{i/o}$-DREADDs in ventral pallidum neurons projecting to the STN in order to selectively and transiently decrease activity of these indirect pathway afferents (Figure 3.2g). Viral expression was largely confined to the portion of the ventral pallidum ventral to the

anterior commissure, with limited expression in the magnocellular preoptic nucleus (Figure 3.2f and h). In experiment 5, a Cre-dependent intersectional vector approach was again used to express $G_{i/o}$-DREADDs, this time in prelimbic cortical neurons projecting to the STN in order to selectively and transiently decrease activity of these hyperdirect pathway afferents (Figure 3.2j). Viral expression was largely confined to the prelimbic cortex, with only a minority of animals showing limited expression in the anterior cingulate or infralimbic cortex (Figure 3.2i and k).

CNO pretreatment alone does not alter baseline locomotion or amphetamine sensitization in DREADD-free rats

Although locomotor sensitization is a valuable model for examining the lasting, sensitizing effects of drugs of abuse (Robinson and Berridge, 1993; White and Kalivas, 1998), off-target effects of experimental pretreatment, such as CNO for activating DREADDs, that alter baseline locomotion can confound sensitization studies. To test for potential off-target behavioral effects of CNO pretreatment, rats pretreated with injections of either 5 mg/kg CNO or saline were sensitized to amphetamine. We found that CNO pretreatment did not alter baseline locomotor activity following saline injections (two-way RM ANOVA, main effect of session $F_{(7, 112)} = 2.516$, $p = 0.019$; no effect of pretreatment $F_{(1, 16)} = 0.842$, $p = 0.842$; no effect of session × pretreatment interaction $F_{(7, 112)} = 0.737$, $p = 0.641$, Figure 3.3a) or the induction of sensitization to amphetamine (two-way RM ANOVA, main effect of session $F_{(7, 161)} = 20.55$, $p < 0.0001$; no effect of pretreatment $F_{(1, 23)} = 0.259$, $p = 0.615$; no effect of session × pretreatment interaction $F_{(7, 161)} = 1.222$, $p = 0.294$, Figure 3.3a) including the acute response to amphetamine during session 1

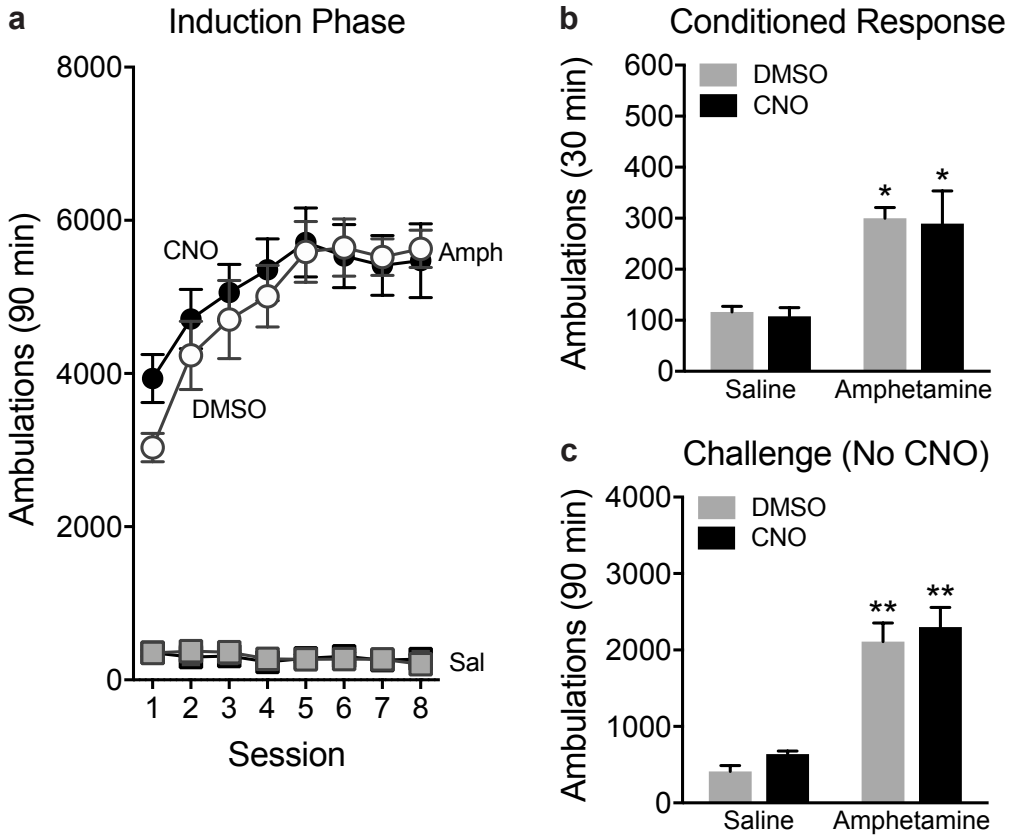

Figure 3.3. There is no difference in baseline locomotion or amphetamine sensitization between CNO- and vehicle-pretreated DREADD-free rats. Data represent mean total ambulations ± SEM during each testing phase by pretreatment/drug or pretreatment history/drug history group. *$p \leq 0.05$, **$p \leq 0.01$. **(a)** Ambulations over 8 locomotor sensitization sessions for CNO and amphetamine treated (black circles), DMSO vehicle and amphetamine treated (white circles), CNO and saline treated (dark gray squares), and DMSO vehicle and saline treated (light grey squares) rats. CNO pretreatment does not significantly alter baseline locomotion in saline treated rats compared to vehicle-saline treated rats, nor does CNO pretreatment alter the induction of amphetamine sensitization compared to vehicle-amphetamine treated rats ($n = 43$). (**b**) Ambulations during conditioned response phase of challenge session by drug history and pretreatment history without additional CNO or amphetamine treatment. Rats with a history of amphetamine treatment showed a conditioned response to drug-associated injection procedures compared to same-pretreatment history saline controls ($n = 15$), and rats with a history of CNO pretreatment during the induction of amphetamine sensitization did not significantly differ from those with a history of vehicle pretreatment in developing a conditioned response. (**c**) Ambulations after amphetamine challenge by drug history and pretreatment history without additional CNO treatment. Rats with a history of amphetamine treatment showed an increased locomotor response compared to same-pretreatment history saline controls ($n = 15$) indicating persistence of sensitization, and

rats with a history of CNO pretreatment during the induction of amphetamine sensitization did not significantly differ from those with a history of vehicle pretreatment.

(Bonferroni; DMSO/amphetamine vs CNO/amphetamine; session 1, $p > 0.789$). After a 15-day withdrawal period, we found no effect of pretreatment history (prior administration of CNO or saline during induction of sensitization) on the development of a conditioned response to the drug-associated injection procedures (two-way ANOVA, main effect of amphetamine history $F_{(1, 11)} = 21.91$, $p = 0.0007$; no effect of pretreatment history $F_{(1, 11)} = 0.0615$, $p = 0.809$; no effect of amphetamine history × pretreatment history interaction $F_{(1, 11)} = 0.0007$, $p = 0.98$, Figure 3.3b) or on the persistence of amphetamine sensitization during a low-dose amphetamine challenge (two-way ANOVA, main effect of amphetamine history $F_{(1, 11)} = 55.19$, $p < 0.0001$; no effect of pretreatment history $F_{(1, 11)} = 0.88$, $p = 0.368$; no effect of amphetamine history × pretreatment history interaction $F_{(1, 11)} = 0.007$, $p = 0.933$, Figure 3.3c) indicating that potential off-target effects of 5 mg/kg CNO pretreatments alone are unlikely to alter amphetamine sensitization.

G_q-DREADD stimulation of STN blocks development of amphetamine sensitization and causes mild hyperactivity in amphetamine-free DREADD controls

Previous studies have highlighted the significance of the STN in addiction neurocircuitry and behavior, but its exact functional role remains unclear. To examine the effects of direct STN manipulation on addiction-related behavior, we stimulated STN neurons expressing G_q-DREADDs during the induction of amphetamine sensitization. We found that activating G_q-DREADDs with CNO pretreatment blocked the induction of sensitization to amphetamine (two-way RM ANOVA, main effect of session $F_{(7, 63)} = 2.279$, $p = 0.039$; main effect of pretreatment $F_{(1, 9)} = 10.97$, $p = 0.009$; main effect of session × pretreatment interaction $F_{(7, 63)} = 8.487$, $p < 0.0001$, Figure 3.4a) while also significantly

increasing baseline locomotion in amphetamine-free saline controls compared to vehicle-pretreated saline controls without DREADD activation (two-way RM ANOVA, main effect of session $F_{(7, 42)} = 3.291$, $p = 0.007$; main effect of pretreatment $F_{(1, 6)} = 43.95$, $p = 0.0006$; no effect of session × pretreatment interaction $F_{(7, 42)} = 1.574$, $p = 0.17$, Figure 3.4a). However, no effect was observed on the acute response to amphetamine (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; session 1, $p > 0.999$). Post-hoc analysis revealed a significantly reduced locomotor response to amphetamine in CNO/amphetamine rats compared to vehicle controls on sessions 5-8 (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; session 5-6, $p < 0.05$; session 7, $p = 0.0001$; session 8, $p < 0.0001$) and a significant increase in baseline locomotion in CNO/saline rats compared to vehicle controls for all sessions (Bonferroni; DMSO/saline vs CNO/saline; session 1-3 and 5-8, $p < 0.0001$; session 4, $p = 0.003$). Observations on an adapted 9-point locomotor-stereotypy rating scale by an experimenter blind to treatment conditions indicated that CNO-pretreated saline controls did not display motor stereotypies, but rather increased normal ambulatory or in-place activity relative to vehicle-pretreated saline controls showing longer periods of inactivity or sleep (data not shown). Following a 15-day withdrawal period, we found that prior activation of G_q-DREADDs with CNO during the induction of amphetamine sensitization attenuated the development of a conditioned response to drug-associated injection procedures (two-way ANOVA, no effect of amphetamine history $F_{(1, 15)} = 3.935$, $p = 0.066$; no effect of pretreatment history $F_{(1, 15)} = 1.307$, $p = 0.271$; main effect of amphetamine history × pretreatment history interaction $F_{(1, 15)} = 6.565$, $p = 0.022$, Figure 3.4b). However, post-hoc analysis revealed that while conditioned responding to injection was observed in rats

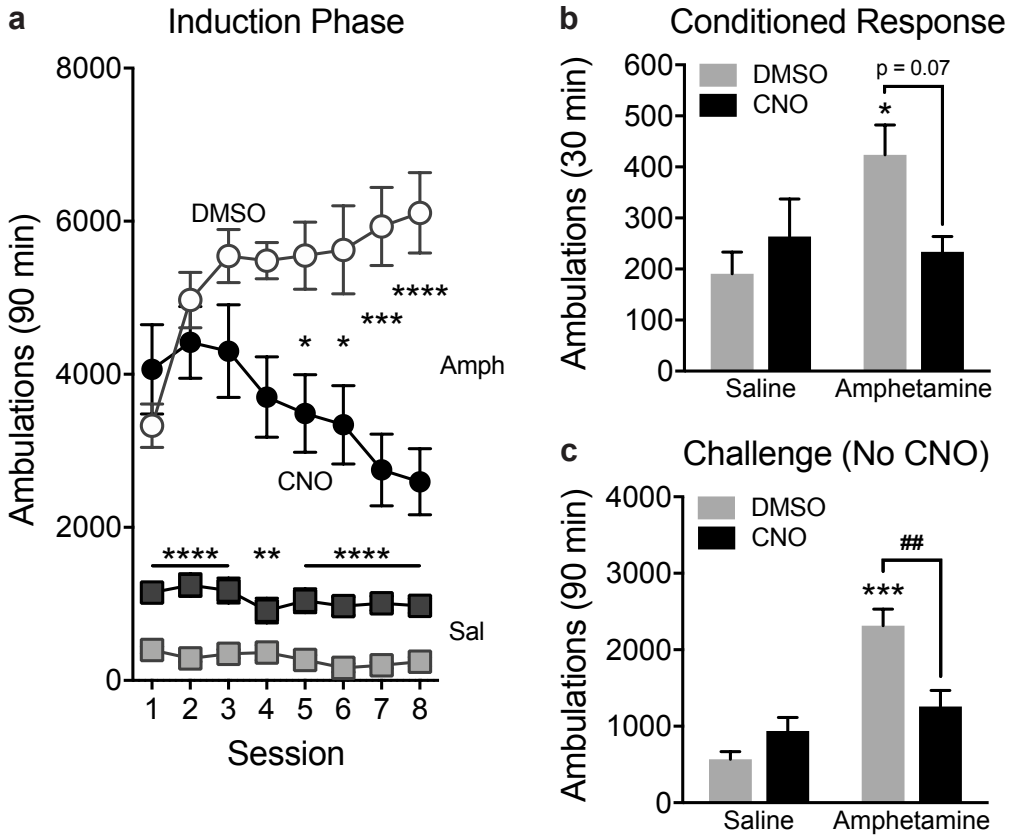

Figure 3.4. Stimulation of STN blocks amphetamine sensitization and causes mild hyperactivity in amphetamine-free DREADD controls. Data represent mean total ambulations ± SEM during each testing phase by pretreatment/drug or pretreatment history/drug history group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$, $^{\#\#}p \leq 0.01$. **(a)** Ambulations over 8 locomotor sensitization sessions for CNO and amphetamine treated (black circles), DMSO vehicle and amphetamine treated (white circles), CNO and saline treated (dark gray squares), and DMSO vehicle and saline treated (light grey squares) rats. CNO pretreatment significantly increases baseline locomotion in saline treated rats (n = 8), and CNO pretreatment blocks the induction of amphetamine sensitization compared to vehicle-amphetamine treated rats (n = 11). (**b**) Ambulations during conditioned response phase of challenge session by drug history and pretreatment history without additional CNO or amphetamine treatment. CNO history prevented development of a conditioned response in amphetamine-history rats, but responding was not significantly different from vehicle-amphetamine-history rats (n = 11). (**c**) Ambulations after amphetamine challenge by drug history and pretreatment history without additional CNO treatment. CNO history blocked persistence of sensitization in amphetamine-history rats. (n = 11).

with vehicle/amphetamine history (Bonferroni; DMSO/saline vs DMSO/amphetamine; p = 0.039), and not rats with CNO/amphetamine history (Bonferroni; CNO/saline vs CNO/amphetamine; $p > 0.999$), the difference in responding between vehicle- and CNO-pretreated amphetamine rats did not reach the level of statistical significance (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; p = 0.073). After administration of a low-dose amphetamine challenge, we found that prior activation of G_q-DREADDs during the induction of amphetamine sensitization also blocked the persistence of sensitization (two-way ANOVA, main effect of amphetamine history $F_{(1, 15)} = 26.32$, $p = 0.0001$; no effect of pretreatment history $F_{(1, 15)} = 2.939$, $p = 0.107$; main effect of amphetamine history × pretreatment history interaction $F_{(1, 15)} = 12.53$, $p = 0.003$, Figure 3.4c), with post-hoc analysis revealing that while rats with vehicle/amphetamine history were significantly sensitized to amphetamine (Bonferroni; DMSO/saline vs DMSO/amphetamine; p = 0.001), rats with CNO/amphetamine history were not (Bonferroni; CNO/saline vs CNO/amphetamine; $p > 0.999$) and showed a significantly reduced response to the amphetamine challenge (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; p = 0.006). This suggests that STN stimulation with G_q-DREADDs not only blocks the expression of amphetamine sensitization during induction, but also prevents its development as measured by an amphetamine challenge, as well as the formation of a conditioned response to drug-associated injection procedures.

$G_{i/o}$-DREADD inhibition of STN enhances induction, but not persistence, of amphetamine sensitization

To further study the effects of direct, bidirectional STN manipulation on addiction-related behavior, we inhibited STN neurons expressing $G_{i/o}$-DREADDs during the

induction of amphetamine sensitization. We found that activating $G_{i/o}$-DREADDs with CNO pretreatment enhanced the induction of sensitization to amphetamine (two-way RM ANOVA, main effect of session $F_{(7, 126)} = 15.7$, $p < 0.0001$; main effect of pretreatment $F_{(1, 18)} = 8.488$, $p = 0.009$; no effect of session × pretreatment interaction $F_{(7, 126)} = 1.372$, $p = 0.223$, Figure 3.5a) without altering the acute response to amphetamine (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; session 1, $p > 0.999$) or baseline locomotion in amphetamine-free saline controls compared to vehicle-pretreated saline controls without DREADD activation (two-way RM ANOVA, main effect of session $F_{(7, 105)} = 4.948$, $p < 0.0001$; no effect of pretreatment $F_{(1, 15)} = 1.353$, $p = 0.269$; no effect of session × pretreatment interaction $F_{(7, 105)} = 1.52$, $p = 0.168$, Figure 3.5a). Post-hoc analysis revealed a significantly enhanced locomotor response to amphetamine in CNO/amphetamine rats compared to vehicle controls on sessions 5-6 (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; session 5, $p = 0.024$; session 6, $p = 0.007$). Following a 15-day withdrawal period, we found that prior activation of $G_{i/o}$-DREADDs with CNO during the induction of amphetamine sensitization did not significantly alter the development of a conditioned response to drug-associated injection procedures (two-way ANOVA, main effect of amphetamine history $F_{(1, 33)} = 22.95$, $p < 0.0001$; no effect of pretreatment history $F_{(1, 33)} = 1.192$, $p = 0.283$; no effect of amphetamine history × pretreatment history interaction $F_{(1, 33)} = 0.002$, $p = 0.969$, Figure 3.5b) or the persistence of sensitization (two-way ANOVA, main effect of amphetamine history $F_{(1, 31)} = 93.85$, $p < 0.0001$; no effect of pretreatment history $F_{(1, 31)} = 0.731$, $p = 0.399$; no effect of amphetamine history × pretreatment history interaction $F_{(1, 31)} = 0.012$, $p = 0.913$, Figure 3.5c), suggesting that the observed enhancement of amphetamine sensitization during

$G_{i/o}$-DREADD-mediated STN inhibition may not persist long after STN inhibition or the induction of sensitization.

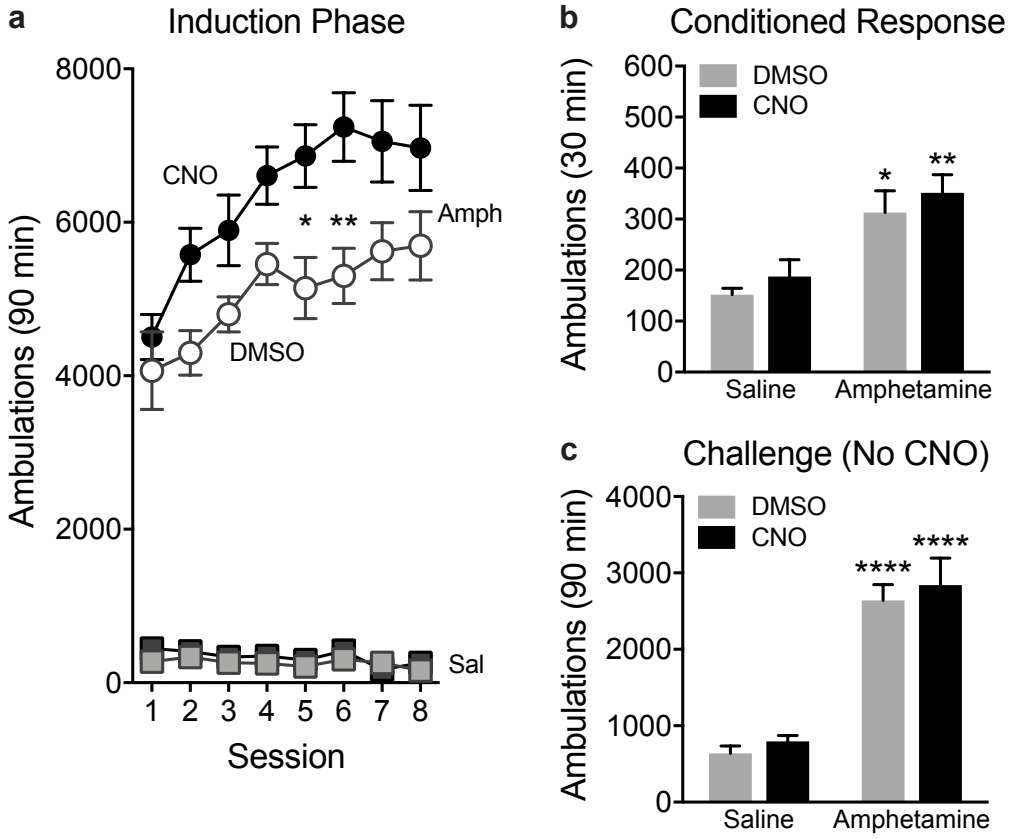

Figure 3.5. Inhibition of STN enhances induction, but not persistence, of amphetamine sensitization. Data represent mean total ambulations ± SEM during each testing phase by pretreatment/drug or pretreatment history/drug history group. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$. **(a)** Ambulations over 8 locomotor sensitization sessions for CNO and amphetamine treated (black circles), DMSO vehicle and amphetamine treated (white circles), CNO and saline treated (dark gray squares), and DMSO vehicle and saline treated (light grey squares) rats. CNO pretreatment enhances the induction of amphetamine sensitization compared to vehicle-amphetamine treated rats (n = 20). (**b**) Ambulations during conditioned response phase of challenge session by drug history and pretreatment history without additional CNO or amphetamine treatment. CNO history did not alter development of a conditioned response in amphetamine history rats (n = 20). (**c**) Ambulations after amphetamine challenge by drug history and pretreatment history without additional CNO treatment. CNO history did not alter persistence of sensitization in amphetamine-history rats. (n = 18).

$G_{i/o}$-DREADD inhibition of STN afferents from ventral pallidum blocks development of a drug-associated conditioned response

While previous studies have directly manipulated STN activity during behavior, few have examined the role of discrete STN afferents, particularly in the context of addiction-related behavior. In order to further investigate the role of STN afferents, we inhibited afferents originating in the ventral pallidum, a major source of indirect pathway input, to test if inhibiting the largely GABAergic input to STN would disinhibit STN neurons and recapitulate results similar to those seen with STN stimulation. This was done using an intersectional viral vector approach to express Cre-dependent $G_{i/o}$-DREADDs only in neurons projecting from the ventral pallidum to STN, which could then be selectively inhibited with CNO. We found that, unlike direct STN stimulation, inhibiting STN afferents from the ventral pallidum did not significantly alter the induction of sensitization to amphetamine (two-way RM ANOVA, main effect of session $F_{(5, 75)} = 26.14$, $p < 0.0001$; no effect of pretreatment $F_{(1, 15)} = 0.204$, $p = 0.658$; no effect of session × pretreatment interaction $F_{(5, 75)} = 0.403$, $p = 0.845$, Figure 3.6a), nor did it affect baseline locomotion in amphetamine-free saline controls (two-way RM ANOVA, main effect of session $F_{(5, 40)} = 3.468$, $p = 0.011$; no effect of pretreatment $F_{(1, 8)} = 0.26$, $p = 0.624$; no effect of session × pretreatment interaction $F_{(5, 40)} = 0.379$, $p = 0.861$, Figure 3.6a). However, like STN stimulation, inhibition of these afferents during the induction of amphetamine sensitization did block the development of a conditioned response to drug-associated injection procedures (two-way ANOVA, main effect of amphetamine history $F_{(1, 23)} = 8.601$, $p = 0.008$; main effect of pretreatment history $F_{(1, 23)} = 7.578$, $p = 0.011$; main effect of amphetamine history × pretreatment history interaction $F_{(1, 23)} = 5.411$, $p = 0.029$, Figure

3.6b), with post-hoc analysis showing no conditioned response in rats with a CNO/amphetamine history (Bonferroni; CNO/saline vs CNO/amphetamine; $p > 0.999$) and a significant difference in responding between vehicle- and CNO-pretreated

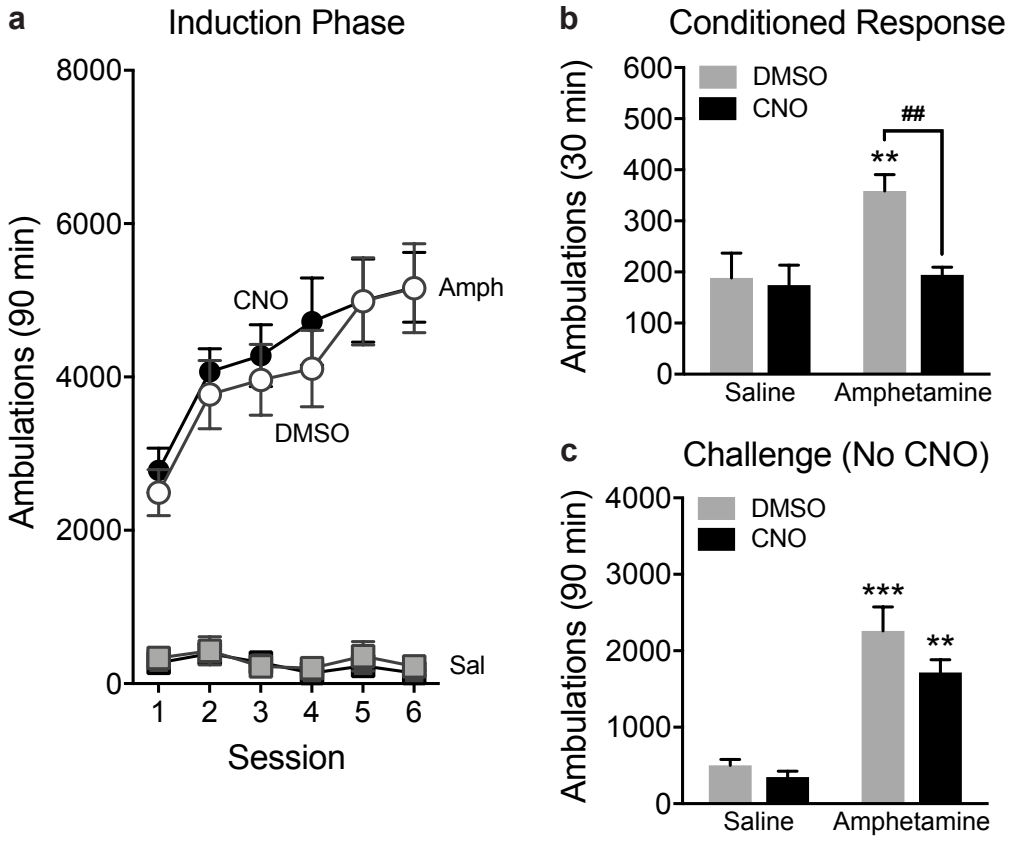

Figure 3.6. Inhibition of STN afferents from ventral pallidum blocks development of a conditioned response. Data represent mean total ambulations ± SEM during each testing phase by pretreatment/drug or pretreatment history/drug history group. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{\#\#}p \leq 0.01$. **(a)** Ambulations over 6 locomotor sensitization sessions for CNO and amphetamine treated (black circles), DMSO vehicle and amphetamine treated (white circles), CNO and saline treated (dark gray squares), and DMSO vehicle and saline treated (light grey squares) rats. CNO pretreatment does not alter the induction of amphetamine sensitization compared to vehicle-amphetamine treated rats (n = 17). (**b**) Ambulations during conditioned response phase of challenge session by drug history and pretreatment history without additional CNO or amphetamine treatment. CNO history blocked development of a conditioned response in amphetamine history rats (n = 17). (**c**) Ambulations after amphetamine challenge by drug history and pretreatment history without additional CNO treatment. CNO history did not alter persistence of sensitization in amphetamine-history rats. (n = 17).

amphetamine rats (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; $p = 0.002$). These group differences were not observed during a low-dose amphetamine challenge (two-way ANOVA, main effect of amphetamine history $F_{(1, 23)} = 45.95$, $p < 0.0001$; no effect of pretreatment history $F_{(1, 23)} = 2.275$, $p = 0.145$; no effect of amphetamine history × pretreatment history interaction $F_{(1, 23)} = 0.717$, $p = 0.406$, Figure 3.6c), suggesting that STN afferents originating in the ventral pallidum may be important for the development of drug-cue associations, but do not strongly modulate the induction or persistence of drug sensitization.

$G_{i/o}$-DREADD inhibition of STN afferents from prelimbic cortex attenuates persistence of amphetamine sensitization and development of a drug-associated conditioned response

The role of hyperdirect pathway projections from the cortex to STN remains poorly understood, especially in the context of addiction-related behavior. Given the importance of the prelimbic cortex in addiction neurocircuitry, we investigated the role of STN afferents originating in prelimbic cortex using the same intersectional viral vector approach used to target afferents from the ventral pallidum to express Cre-dependent $G_{i/o}$-DREADDs only in neurons projecting from prelimbic cortex to STN. Since hyperdirect pathway input to the STN is thought to be primarily glutamatergic, we hypothesized that inhibition of that input might recapitulate results similar to those of direct STN inhibition. However, inhibiting STN afferents from prelimbic cortex did not significantly alter the induction of sensitization to amphetamine (two-way RM ANOVA, main effect of session $F_{(5, 100)} = 25.32$, $p < 0.0001$; no effect of pretreatment $F_{(1, 20)} = 0.075$, $p = 0.787$; no effect of session × pretreatment interaction $F_{(5, 100)} = 1.243$, $p = 0.295$, Figure 3.7a), nor did it

alter baseline locomotion in amphetamine-free saline controls (two-way RM ANOVA, no effect of session $F_{(5, 45)}$ = 2.133, p = 0.079; no effect of pretreatment $F_{(1, 9)}$ = 0.224, p = 0.647; no effect of session × pretreatment interaction $F_{(5, 45)}$ = 1.061, p = 0.395, Figure 3.7a). Surprisingly, we found that inhibition of these hyperdirect pathway afferents during the induction of amphetamine sensitization did block the development of a conditioned response to drug-associated injection procedures (two-way ANOVA, main effect of amphetamine history $F_{(1, 29)}$ = 5.412, p = 0.027; main effect of pretreatment history $F_{(1, 29)}$ = 3.522, p = 0.071; main effect of amphetamine history × pretreatment history interaction $F_{(1, 29)}$ = 6.622, p = 0.016, Figure 3.7b), with post-hoc analysis showing no conditioned response in rats with a CNO/amphetamine history (Bonferroni; CNO/saline vs CNO/amphetamine; p > 0.999) and a significant difference in responding between vehicle- and CNO-pretreated amphetamine rats (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; p = 0.003). In addition, prior inhibition of these hyperdirect pathway afferents significantly attenuated the persistence of amphetamine sensitization (two-way ANOVA, main effect of amphetamine history $F_{(1, 29)}$ = 47.78, p < 0.0001; main effect of pretreatment history $F_{(1, 29)}$ = 7.534, p = 0.01; main effect of amphetamine history × pretreatment history interaction $F_{(1, 29)}$ = 4.185, p = 0.05, Figure 3.7c), with post-hoc analysis showing a significant difference in response to the amphetamine challenge between vehicle- and CNO-pretreated amphetamine rats (Bonferroni; DMSO/amphetamine vs CNO/amphetamine; p = 0.007). These data, which more closely resemble direct stimulation of STN in terms of conditioned responding and persistence of drug sensitization, may suggest a more complex role for how the STN integrates different afferent inputs, perhaps with some inputs, like those of the hyperdirect pathway,

synapsing first onto GABAergic interneurons or differentially modulating either discrete subpopulations of STN neurons with distinct functions or modulating oscillatory activity within the STN.

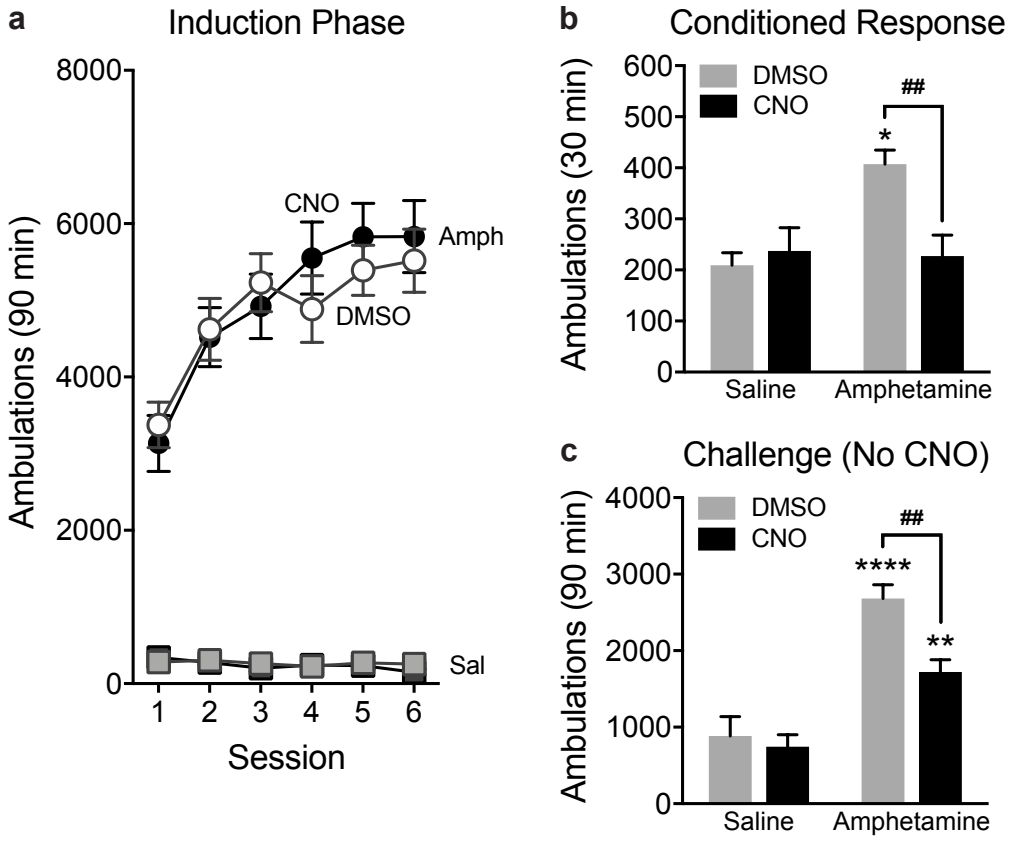

Figure 3.7. Inhibition of prelimbic cortex-STN projections attenuates persistence of amphetamine sensitization and development of a conditioned response. Data represent mean total ambulations ± SEM during each testing phase by pretreatment/drug or pretreatment history/drug history group. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$, $^{\#\#}p \leq 0.01$. **(a)** Ambulations over 6 locomotor sensitization sessions for CNO and amphetamine treated (black circles), DMSO vehicle and amphetamine treated (white circles), CNO and saline treated (dark gray squares), and DMSO vehicle and saline treated (light grey squares) rats. CNO pretreatment does not alter the induction of amphetamine sensitization compared to vehicle-amphetamine treated rats (n = 22). (**b**) Ambulations during conditioned response phase of challenge session by drug history and pretreatment history without additional CNO or amphetamine treatment. CNO history blocked development of a conditioned response in amphetamine history rats (n = 22). (**c**) Ambulations after amphetamine challenge by drug history and pretreatment history without additional CNO treatment. CNO history attenuated persistence of sensitization in amphetamine-history rats. (n = 22).

3.5 Discussion

Locomotor sensitization to drugs results from the enhancement of psychomotor activating and incentive motivational drug effects following repeated drug exposure, representing lasting alteration of the neural circuits underlying addiction (Paulson and Robinson, 1991; Robinson and Becker, 1986; Robinson and Berridge, 2008; Segal and Mandell, 1974; Wise and Bozarth, 1987). Here we show that stimulation of STN neurons with G_q-DREADDs robustly blocks the development and persistence of amphetamine sensitization. In addition, G_q-DREADD stimulation of STN induces mild hyperactivity in amphetamine-free DREADD controls. In contrast, inhibition of STN neurons with $G_{i/o}$-DREADDs transiently enhances the induction of amphetamine sensitization without altering conditioned responding to drug-associated injection procedures or persistence of sensitization. Inhibition of STN neurons also has no effect on the acute locomotor response to amphetamine, nor does it alter locomotor activity in amphetamine-free controls. Inhibition of afferents from ventral pallidum has no significant effect on the induction or persistence of amphetamine sensitization, however, this manipulation does block the development of conditioned responding to injection procedures. Surprisingly, inhibition of afferents from prelimbic cortex has no effect on the induction of sensitization, but attenuates both conditioned responding to injection and persistence of sensitization.

First, the mild hyperactivity observed after G_q-DREADD stimulation of STN suggests the manipulation may have altered neuronal processes other than drug sensitization, potentially confounding the results of that experiment given its reliance on locomotor output as a proxy for drug-induced plasticity. However, it should be noted that the CNO-amphetamine treated rats did not express a hyperactive phenotype, but rather

showed dramatically decreased locomotion compared to vehicle-amphetamine controls, suggesting that the primary changes observed in locomotor output were not entirely due to the same mechanism that triggered mild hyperactivity in saline-CNO controls. Alternatively, it is possible that such significant disruption of STN activity led to a motor deficit that both reduced locomotion in CNO-amphetamine animals and disrupted normal, quiescent activity in CNO-saline controls, thus generating a hyperactive phenotype while also blocking the expression of locomotor sensitization. This, however, also seems unlikely given that CNO-amphetamine animals showed no conditioned response to drug-associated injection or persistence of amphetamine sensitization long after administration of CNO during sensitization induction, suggesting that sensitization was not merely masked in its expression, but fully blocked by STN stimulation.

On one hand, the results from our direct STN manipulations fit well with previous examinations of drug sensitization following STN lesion (Uslaner *et al*, 2005), where inhibition of STN activity by excitotoxic lesion also enhanced cocaine sensitization. Along with the disruption of amphetamine sensitization seen after G_q-DREADD stimulation, these data also fit the classical view of the striatal direct and indirect pathways, where inhibition of STN and therefore indirect pathway activity might be expected to enhance addiction-related behavior while excitation of STN and indirect pathway should interrupt such behavior (Lobo and Nestler, 2011; Wall *et al*, 2013; Yager *et al*, 2015). However, this simplified model does not account for the many anti-addictive behavioral effects previously observed after STN lesion (Baunez *et al*, 2005; Pelloux and Baunez, 2017) or inhibition (Bentzley and Aston-Jones, 2016; Pelloux *et al*, 2018; Rouaud *et al*, 2010; Wade *et al*, 2017).

As mentioned previously, these discrepancies could result from a number of factors. One explanation may lie in the ability of incomplete lesions to induce functional and morphological reorganization in surviving brain tissue (Castro-Alamancos and Borrel, 1995). Chemogenetic manipulation provides a clear advantage here, given its less invasive and more reversible impact on target neurons. While this potential reorganization might make the effects of lesions somewhat unpredictable, this does not account for discrepancies which arise from studies using non-lesion methods including DBS, and even those utilizing excitotoxic lesions of STN describe complete ablation of targeted neurons. The specific patterning of STN activity, particularly neuronal beta band oscillations, may also be a critical component of STN function and reason for paradoxical results of direct STN manipulation, with maladaptive PD- (Brown *et al*, 2001; Eusebio *et al*, 2012; Limousin *et al*, 1995) and drug-related (Pelloux *et al*, 2018) behaviors occurring alongside abnormal oscillatory activity in the STN, which is disrupted by therapeutic STN DBS and lesions (Eusebio *et al*, 2012; Limousin *et al*, 1995; Pelloux *et al*, 2018). This patterning could be unpredictably altered by lesions, or variably reshaped by DBS based on electrode placement and the dynamics of local microcircuitry. Chemogenetic manipulation might also exert variable effects on patterned STN activity, however, the engagement of endogenous intracellular signaling mechanisms by DREADDs might make it a more physiologically relevant means of modulating the STN. The potential for partial lesioning or variable distance of STN neurons from DBS electrodes also presents additional complications for STN studies because human, nonhuman primate, and rat STN is thought to be topographically subdivided into motor, associative, and limbic segments defined by their afferent connections (Alkemade, 2013; Haynes and Haber,

2013; Kita *et al*, 2014; Temel *et al*, 2005). This suggests that both the location and reach of STN manipulations could variably influence behavioral outcomes by disrupting separate functional territories within STN, and similar localization problems may affect experiments utilizing pharmacological manipulation with microinfusion of drugs such as muscimol. While irregular viral expression of DREADDs in STN could also exert similar effects, the limited signal amplification achieved by engaging intracellular signaling cascades may help equalize the magnitude of DREADD modulation between neurons with low and high DREADD expression. Along these lines, it is also possible that the conflicting data may be a product of STN manipulations differentially impacting specialized, segregated information streams within the STN that engage distinct neuronal subpopulations responsive to separate reinforcers. There is indeed evidence that subpopulations of STN neurons may at any given time encode separate rewards (such as cocaine vs sucrose), along with reward magnitude and omission (Breysse *et al*, 2015; Lardeux *et al*, 2009; 2013). Inconsistent targeting of neuronal subpopulations with distinct afferent or efferent connections that both partially integrate and partially segregate separate streams of information could contribute to varied behavioral outcomes. For future experiments directly manipulating STN, it may prove useful to pair manipulations with electrophysiological or calcium imaging-based analysis of STN activity to better understand how a given manipulation alters STN activity in real time. These complications also highlight the need for studies that utilize reversible, spatially precise methods to functionally dissect STN afferents that may interact with STN neuronal subpopulations in more physiologically relevant ways, thus avoiding some of the problems of direct STN manipulation while also helping to map functions of STN circuitry.

To this end, we sought to modulate two critical components of STN input using Cre-dependent $G_{i/o}$-DREADDs. Based on previous studies, we hypothesized that inhibition of afferents from ventral pallidum might attenuate amphetamine sensitization and conditioned responses to drug-associated injection procedures much as disconnection reduces reinstatement and reacquisition of alcohol seeking, in addition to reducing motivation for alcohol (Prasad and McNally, 2016). Surprisingly, no effect of this inhibition was observed on the induction of sensitization, however, inhibiting afferents from ventral pallidum did appear to block the development of a conditioned response to drug-associated injection. This could suggest that ventral pallidum-STN projections are important for regulating responsiveness to drug-associated cues, which in turn could affect reinstatement and reacquisition of drug seeking behavior.

Based on the limited literature characterizing hyperdirect afferents to STN, we hypothesized that inhibition of the glutamatergic prelimbic-STN projection would ultimately reduce STN activity and enhance the development of amphetamine sensitization. Surprising, this manipulation had effects similar to that of inhibiting the GABAergic ventral pallidum-STN projection, blocking the development of a conditioned response to drug-associated injection while also attenuating the persistence of amphetamine sensitization. While these results do not cleanly fit with the classical view of how excitatory inputs to STN might impact behavior, they actually fit well within the context of how prelimbic cortex more generally influences addiction-related behavior. For example, while photoinhibition of prelimbic cortical neurons is known to enhance cocaine self-administration (Martín-García *et al*, 2014), this same manipulation also reduces cocaine-primed reinstatement among rats that have undergone a high-frequency cocaine

intake schedule (Martín-García *et al*, 2014; Stefanik *et al*, 2013). This seeming switch in how prelimbic cortex oppositely regulates addiction-like behavior between self-administration and reinstatement has yet to be fully understood, but it seems possible that projections from prelimbic cortex to STN could play a role. Given the lack of acute effects of prelimbic-STN inhibition during induction of sensitization, it seems possible that changes in prelimbic activity initiate neuroadaptations that only later become obvious through regulation of drug-associated cues. One mechanism for these delayed effects could lie in the heterosynaptic regulation of indirect pathway inputs to STN by cortical hyperdirect afferents. Recent work demonstrates that, under normal conditions, hyperdirect pathway transmission potentiates indirect pathway input to STN, keeping the excitatory and inhibitory effects of each pathway in balance (Chu *et al*, 2015). Under this model, repeated inhibition of hyperdirect inputs to STN from prelimbic cortex might reduce the strength of indirect pathway inputs, ultimately resulting in similar effects between inhibition of the indirect and hyperdirect inputs to STN. Alternatively, it is possible the similarities between these two seemingly opposite manipulations resulted from mere off-target effects of CNO administration, though this is unlikely given that CNO administration in DREADD-free rats had no effect on the development of a conditioned response to drug-associated injections or on the persistence of sensitization.

Taken together, these results suggest the STN and its afferents play more complex roles in the regulation of addiction-like behavior than previously recognized in classic direct and indirect pathway models. In addition, the surprising results yielded by modulation of hyperdirect pathway projections stress the need for further characterization of STN afferents to better understand how STN circuitry regulates complex behaviors like

those associated with addiction. And finally, ambiguities over how these inputs are integrated in STN, specifically with respect to patterned, oscillatory activity, strongly suggest that future studies would benefit from more complex *in vivo* electrophysiological characterization of STN activity or newer *in vivo* imaging methods such as calcium imaging to analyze how distinct inputs modulate clusters of STN neurons.

Chapter 4

Conclusions and Future Directions

The work described in this thesis explored two main components of corticolimbic circuitry through the lens of addiction-related behavior and chemogenetic modulation of discrete neuronal populations. First, I demonstrated how inhibition of corticostriatal projections with Cre-dependent DREADDs could be used to better understand the role of those projections in addiction-like behavior. Next, I utilized a similar chemogenetic approach to reveal how the STN is capable of differentially modulating addiction-like behavior dependent on the engagement of distinct afferent connections. Both instances involved corticolimbic circuitry circumstantially implicated in addiction-related behavior, but not entirely resolved in their function through previous studies lacking the circuit and temporal specificity afforded by chemogenetic tools.

Chapter 2 described in depth how corticostriatal projections from mPFC to NAc are critical for the regulation of addiction-like behavior. Specifically, this study demonstrated how inhibition of the mPFC-NAc pathway with Cre-dependent $G_{i/o}$-DREADDs during amphetamine sensitization attenuated the development of amphetamine sensitization and facilitated a conditioned response to drug-associated injection procedures. This work also revealed how the same chemogenetic manipulation during cocaine self-administration did not alter overall cocaine consumption, but instead impaired extinction learning in the absence of drug availability and later enhanced reinstatement of cocaine-primed drug seeking, while also re-normalizing drug seeking when DREADDs were reactivated. Together, these results indicate that mPFC-NAc

projections exert important, but also somewhat limited, control over addiction-like behavior, and that the effect of manipulating this pathway can vary depending on the behavioral paradigm being used to model addiction.

Previous studies have demonstrated how mPFC may regulate sensitization to addictive drugs (Steketee and Kalivas, 2011; Tzschentke and Schmidt, 2003; Vanderschuren and Kalivas, 2000; Wolf, 1998), and the results reported here fit well with previous work and more generalized models of how glutamatergic signaling regulates these behaviors. While it is somewhat surprising that mPFC-NAc inhibition both attenuated the induction of amphetamine sensitization and enhanced development of a conditioned response, these results fit well with the previously described pattern of mPFC regions switching directionality of effect on addiction-like behavior between initial drug exposure (induction of sensitization in this case) and post-abstinence cue-reactivity (conditioned responding). In particular, these results closely align with previous studies of vmPFC, where inhibition of vmPFC neurons blocks extinction of cocaine CPP only after a 3-week drug withdrawal (Van den Oever *et al*, 2013), but activation of vmPFC attenuates cue-induced reinstatement of cocaine seeking after extinction (Augur *et al*, 2016) and facilitates the extinction of a cocaine conditioned place preference (CPP) (Van den Oever *et al*, 2013). This suggests that extinction-dependent plasticity in the vmPFC is necessary for vmPFC regulation of cue-driven drug seeking, and paired with the more ventral targeting of DREADD expression for this sensitization experiment, it seems possible that our manipulation primarily engaged vmPFC over dmPFC.

In contrast to this, the delayed extinction and mixed reinstatement of cocaine-seeking results under this same manipulation may have resulted from targeting both

dmPFC and vmPFC with DREADDs, and specifically prelimbic cortex. While reinstatement of cocaine seeking was initially elevated in animals with a history of mPFC-NAc inhibition, additional inhibition prior to the reinstatement session reduced reinstatement to normal levels, similar to the reduction in reinstatement observed in previous studies. For instance, photoinhibition of prelimbic cortical neurons is known to reduce cocaine-primed reinstatement (Martín-García *et al*, 2014; Stefanik *et al*, 2013). This contrast with the sensitization data also fits given that viral expression of DREADDs for this experiment was more dorsally located and may have engaged prelimbic cortex over vmPFC. Together, these results may suggest the need for more targeted manipulations of mPFC in future experiments, such that functions of prelimbic vs vmPFC can be more directly compared.

Further, given the ambiguity discussed in Chapter 1 regarding how these cortical subregions are defined both between studies and across species, these results may also highlight the need to further restrict manipulations to cortical populations defined more by larger connectivity than anatomical region. Methods for achieving this remain limited, but future studies could, for instance, utilize related combinatorial viral-vector approaches to restrict manipulations to neurons possessing defined collateral projections by both their downstream target and by collaterals that distinguish them from nearby or intermingled neuronal populations. For example, given that prelimbic, but not infralimbic, neurons project to the claustrum (Vertes, 2004), and that PT-type, but not IT-type, cortical neurons send diverse collaterals to regions including the striatum (Shepherd, 2013) infusion of a Cre-dependent $G_{i/o}$-DREADD construct into mPFC, CAV2-Cre into the claustrum, and infusion of CNO into the striatum should limit DREADD inhibition to circuit-defined PT-

type, prelimbic corticostriatal projections with collateral projections to the claustrum. Whether or not such a restricted manipulation could yield less ambiguous behavioral results remains an open question, and further development of technologies that can address similar questions in a broader set of circuitry is needed.

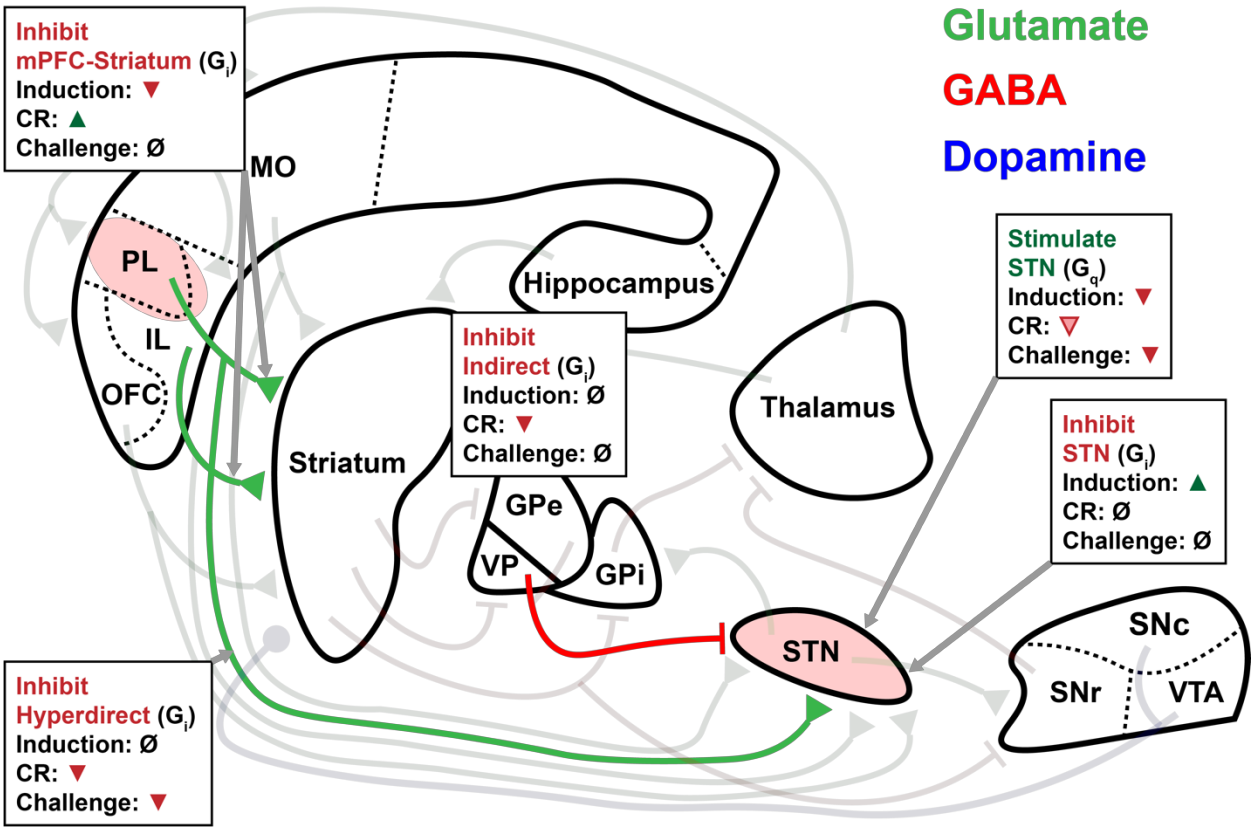

Figure 4.1. Graphical overview of sensitization experiments and corticolimbic circuitry. Pink regions represent approximate anatomical areas targeted by DREADD manipulations in sensitization experiments. Red triangles represent a significant decrease ($p \leq 0.05$) in specified behavior among DREADD-activated animals compared to vehicle controls without DREADD activation. Pink triangles represent a marginal decrease ($p \leq 0.1$) in specified behavior among DREADD-activated animals compared to vehicle controls. Green triangles represent a significant increase ($p \leq 0.05$) in specified behavior among DREADD-activated animals compared to vehicle controls. ∅ represents no change in behavior compared to vehicle controls.

Chapter 3 of this thesis detailed how the same chemogenetic strategies described above could be utilized for characterizing STN circuitry in the context of addiction. Because the STN has so rapidly gained attention as a potential therapeutic target for

addiction treatment (Baunez *et al*, 2005; Bentzley and Aston-Jones, 2016; Luigjes *et al*, 2012; Pelloux and Baunez, 2013; 2017; Pelloux *et al*, 2018; Pierce and Vassoler, 2013; Uslaner *et al*, 2005) despite a paucity of literature detailing its functional connectivity, studies better characterizing the STN and its afferents seem necessary for the advancement of STN modulation as a viable addiction therapy. To this end, we used DREADDs to bidirectionally manipulate STN and found that STN stimulation robustly blocked the development of amphetamine sensitization while inhibition only transiently enhanced its induction. In order to dissect more physiologically relevant functions of STN, we also inhibited afferent projections from ventral pallidum and prelimbic cortex. Surprisingly, both manipulations blocked the development of a conditioned response to drug-associated injections, while only inhibition of afferents from prelimbic cortex attenuated persistence of amphetamine sensitization. While these results fit well with previous work examining disconnection of ventral pallidum and STN (Prasad and McNally, 2016), as well as direct manipulation of prelimbic cortex (Martín-García *et al*, 2014; Stefanik *et al*, 2013), the similarity in effects of these two manipulations was surprising given that one is an inhibitory GABAergic projection and the other is a stimulatory glutamatergic projection. One explanation for these similar effects may lie in the heterosynaptic regulation of indirect pathway inputs to STN by cortical hyperdirect afferents. It is now known that, under normal conditions, hyperdirect pathway transmission potentiates indirect pathway input to STN through heterosynaptic long term potentiation (hLTP), keeping the excitatory and inhibitory effects of each pathway in balance (Chu *et al*, 2015). This model may suggest that repeated inhibition of hyperdirect inputs to STN from prelimbic cortex could reduce the strength of indirect pathway inputs,

ultimately resulting in similar effects between inhibition of the indirect and hyperdirect inputs to STN. Alternatively, these surprising results could stem from some of the same complexities surrounding direct manipulations of STN and the paradoxical results they generate, including inconsistent targeting of STN neuronal subpopulations with variable reward response properties (Breysse *et al*, 2015; Lardeux *et al*, 2009; 2013), and differential modulation of patterned STN activity, including neuronal beta band oscillations implicated in disordered behavior (Brown *et al*, 2001; Eusebio *et al*, 2012; Limousin *et al*, 1995; Pelloux *et al*, 2018). This last point in particular stands out as a major barrier to fully characterizing the STN, since chemo/optogenetic tools alone cannot resolve how distinct STN inputs are integrated into complex patterns of synchronized and unsynchronized activity. Addressing this question will almost certainly require future studies utilizing the latest advancements in *in vivo* electrophysiology, as well as *in vivo* calcium imaging. The latter in particular holds great promise for better understanding how patterned STN activity is shaped, perhaps by imaging clusters of neurons in parallel with manipulations of specific STN afferents or addiction-related behavioral tasks.

In conclusion, this thesis provides a framework for understanding how corticostriatal and subthalamic circuits regulate addiction-related behavior. Furthermore, it draws parallels between the two circuits in terms of how cortical regions frequently switch between pro-addictive and anti-addictive functions dependent on drug abstinence and other experimental parameters. Finally, this work leaves open several new avenues for future work, particularly in characterizing STN afferents and how they are integrated to regulate behavior, which may one day improve therapeutics for treating addiction.

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Yager LM, Garcia AF, Donckels EA, Ferguson SM (2019). Chemogenetic inhibition of direct pathway striatal neurons normalizes pathological, cue-induced reinstatement of drug-seeking in rats. *Addiction Biology* **24**: 251–264.
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Education

2013 - 2019
Neuroscience (PhD)
University of Washington

2009 - 2013
Psychology (BA)
Neurobiology, Physiology & Behavior (BS)
University of California, Davis

Experience

Predoctoral Research Associate – Seattle Children's Research Institute
Susan M. Ferguson Lab
September 2013 - June 2019

Studied the corticothalamic-basal ganglia network of the brain using cutting-edge molecular pharmacology and multiplex behavioral models to better understand the regulation of compulsive behaviors associated with drug addiction and potentially aid treatment development. Directly mentored two undergraduate research assistants and served as a laboratory TA for NBIO 301 - Introduction to Cellular and Molecular Neurobiology.

Undergraduate Research Assistant – University of California, Davis
Brian C. Trainor Lab
April 2011 - June 2013

Investigated the effects of chronic stressors on a transgenic mouse model of ErbB2/Her2-positive breast cancer and received the UC Davis President's Undergraduate Fellowship to study the role of neuropeptide Y in resilience to chronic social stress.

Volunteer / Committee Positions

Officer – UW Neuroscience Community Outreach Group
2014 - Present

Organized and ran outreach ranging from class workshops to large 500-1000 person events. Created and implemented Common Core-aligned lesson plans teaching

basic neuroscience to all grade levels that are freely distributed to teachers/utilized at classroom workshops. Worked to increase engagement in educational outreach within UW Neuroscience and refocus outreach efforts on local schools underserved by STEM outreach.

Admissions Committee Member – UW Graduate Program in Neuroscience
2015 - 2018

Performed candidate review, applicant outreach, student volunteer coordination, event planning/transportation logistics, and work on initiatives aimed at recruiting members of historically underrepresented minorities. Worked to preserve best practices during transition to new program directorship and critically analyzed factors that led to successful student recruitment.

Neuroscience Program Contact – UAW Local 4121
2017 - 2018

Facilitated communication between union leadership and students, ran orientations, bolstered union engagement and membership.

Awards and Honors

Neuroscience Accelerator Award – University of Washington – 2017
Graduate Opportunity Program Award – University of Washington GO-MAP – 2013
President's Undergraduate Fellowship – University of California, Davis – 2012
United Health Foundation Diverse Scholar – United Health Foundation – 2010

Peer-Reviewed Publications

Garcia AF, **Nakata KG**, Ferguson SM (2018). Viral strategies for targeting cortical circuits that control cocaine-taking and cocaine-seeking in rodents. Pharmacol Biochem Behav 174: 33-41

Kerstetter KA, Wunsch AM, **Nakata KG**, Donckels E, Neumaier JF, Ferguson SM (2015). Corticostriatal afferents modulate responsiveness to psychostimulant drugs and drug-associated stimuli. Neuropsychopharmacol 41: 1128-1137

Poster Presentations and Abstracts

Nakata KG, Yin E, Ferguson SM (2017). DREADD-mediated modulation of the subthalamic nucleus alters locomotor sensitization to amphetamine in rats. Society for Neuroscience, Washington D.C.