Characterizing the role of cortical neuronal subtypes and drug intake pattern in cocaine addiction

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Abstract

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Drug addiction is a complex neuropsychiatric disorder that takes a great emotional and financial toll on those who suffer from it as well as society as a whole. Psychologically, drugs of abuse produce an unusual mix of symptoms characterized by euphoric reward states and the desire to seek more drug as well as aversive states characterized by dysphoria and physical discomfort. It is of great importance to understand how drugs of abuse are able to induce these different psychological symptoms and how these components combine to create a disease that is chronic with high rates of recidivism. The corticolimbic circuitry is composed of several nuclei that are involved in motivated and affective behaviors under normal conditions. During drug use, many molecular and synaptic adaptations occur in this circuit, which is believed to underlie the transition to a compulsive drug use state.

In order to study drug addiction effectively, it must first be effectively modeled in animals. In the first chapter, I describe an intermittent access self-administration paradigm that captures many aspects considered crucial to compulsive drug use. Intermittent access self-administration produces a coherent behavioral variability that allows for the separation of animals into compulsive and non-compulsive groups. Compulsive animals demonstrate an enhanced sensitization to cocaine, an increased susceptibility to cue-induced reinstatement, escalation of their drug intake over time, and a change in seeking patterns that are indicative of binge-type behaviors. Continuous access self-administration, while producing animals that individually score highly on individual aspects of addiction behaviors, fails to produce a group of compulsive animals that score highly across many different measures.

In chapter 2 and 3, we begin to characterize the contributions of striatum-projecting anterior cingulate cortical (ACC) neurons in a cell-type specific manner. Although corticostriatal projections play an important role in addiction, no attempts have been made to study the contributions of these projections in a manner that accounts for cortical cell-type specificity. The cortex is composed of two striatal projection populations (IT and PT-type neurons), which differ in a multitude of ways. First, we show that IT and PT-type neurons selectively participate in the negative and positive aspects of cocaine use, respectively. Next, we demonstrate that activity in PT-type neurons is not inherently rewarding and does not contribute to the experience of hedonic natural rewards. Finally, we show that inhibition of PT-type neurons in the ACC transiently enhances cocaine-induced locomotor sensitization, but does not affect the motivation to obtain drug or seek drug on progressive ratio and reinstatement tests.

Dedicated to:

This thesis is dedicated to my family, especially my parents. Everything that I achieve or accomplish is a reflection of the support, companionship, and care that I have received from loved ones.

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Acknowledgments

"The world is so empty if one thinks only of mountains, rivers, and cities; but to know someone who thinks and feels with us, and who, though distant, is close to us in spirit – this can make life into a garden."

- *Johann von Goethe*

Graduate school and performing the research that formed the basis of this thesis has, at times, been incredibly challenging. Sometimes I wonder if the adversity posed during this period are special to graduate school or a reflection of deeper personal challenges that everyone faces around this time in their life regardless of profession or location. What I am certain of, is that I could not succeed or grow without the support and encouragement of the people in my life. They have not only allowed me to succeed, they have turned my world into a garden, and for that I will forever be grateful.

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Chapter 1

Introduction

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

A. Cocaine Addiction

Drug addiction is a major societal issue that carries with it devastating personal, social, and economic implications. The National Survey on Drug Use and Health estimated that there were approximately 1.5 million current cocaine users in the United States in 2014. Pharmacologically, cocaine primarily acts at the dopamine transporter causing it to flood the synapse with dopamine (Ritz et al., 1987). It can have widespread effects throughout the corticolimbic circuitry, though (Nestler, 2001; Everitt et al., 2008). The psychological effects of cocaine addiction are complicated. It is perhaps best known for the euphoric “rush” that it causes; however, it can also cause a number of negative symptoms related to anxiety, panic, and dysphoria (Gay 1982; Ettenburg and Geist, 1991; Washton and Gold, 1984; Anthony et al., 1989; Trinkoff et al., 1990; Wood et al., 2014).

Over time as casual use transitions to compulsive use, it is characterized by an escalation of intake, an inability to stop taking drugs despite negative consequences, loss of control over drug-seeking leading to binge-like behaviors, sensitization of craving, and a chronic susceptibility to relapse. Many behavioral models now exist to probe these different aspects of drug use. For instance, repeated injections of psychostimulants produce progressively greater locomotor responses, a process known as locomotor sensitization. Since these changes are long-lasting and motor circuits overlap heavily with motivational circuits, this is a useful paradigm for testing sensitization of craving (Robinson and Berridge, 2000; White and Kalivas, 2008). Drug self-administration models can be used to test escalation of intake and susceptibility to relapse (Ahmed and Koob, 1998; McFarland and Kalivas, 2001). More simply, cocaine

has rewarding properties sufficient to produce a conditioned place preference, but also aversive properties that can produce a conditioned taste aversion, making these tests valuable tools for probing the positive and negative components of cocaine (Tzschentke 2007; Hunt & Amit, 1987; Ferrari et al., 1991).

B. Prefrontal Cortex Composition

The prefrontal cortex (PFC) is primarily made up of excitatory glutamatergic cells (pyramidal and spiny stellate cells), which project both within and outside of the cortex and are found in all cortical layers except layer I (Jones, 1984, Staiger et al., 2004, Kubota et al., 2016, Tremblay et al., 2016). In addition, there is a small population (10–15%) of GABAergic interneurons (Meyer et al., 2011). Approximately 40% of these interneurons are parvalbumin (PV) positive, 30% are somatostatin (SOM) positive, and 30% express the 5-HT3a serotonin receptor (Tremblay et al., 2016). These interneuron classes can also be subdivided further based on morphology, physiology, and connectivity patterns. Despite their relatively small population numbers, these interneurons are located in all cortical layers and can make substantial connections within cortex, allowing them to play a critical role in modulating cortical output and function (Kubota et al., 2016, Tremblay et al., 2016).

Layers of cortex

The cortex is made up of six layers. Layer I is largely composed of fibers; however, there are also GABAergic interneurons in this layer, which can modulate the fibers that pass through it (Garcia-Munoz and Arbuthnott, 2015). Layers II/III are primarily made up of pyramidal cells and receive intra-cortical input as well as input from thalamus. These layers provide significant input to the deep layers of cortex (Kuroda et

al., 1998, Kubota et al., 2016, Hirai et al., 2012, Kim et al., 2015). Layer IV is composed mostly of corticocortical spiny stellate and pyramidal cells that receive input from the thalamus (Staiger et al., 2004). Layers V and VI contain the subcortically projecting pyramidal neurons; although many of these neurons also project within cortex. Layer V neurons have strong projections to the striatum and some thalamic projections whereas Layer VI neurons tend to project to thalamus but not striatum (Thomson, 2010, Kubota et al., 2016, Hirai et al., 2012, Kim et al., 2015, Wilson, 1987, Reiner et al., 2010, Shepherd, 2013, Morishima and Kawaguchi, 2006).

Regions of PFC

The medial prefrontal cortex (mPFC) is typically divided into two major divisions, the dorsal medial (dmPFC) and the ventromedial (vmPFC) (Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007, Moorman et al., 2015, Vertes, 2004). In rodents, the dmPFC consists of the medial agranular, anterior cingulate, and dorsal aspect of prelimbic cortex and the vmPFC includes the ventral portion of prelimbic cortex and the infralimbic cortex (Berendse and Groenewegen, 1991, Heidbreder and Groenewegen, 2003, Vertes, 2006, Hoover and Vertes, 2007). The dmPFC is typically considered to be more involved with motor tasks than the vmPFC, which is believed to play a larger role in emotion and affective behaviors due to its significant connections with limbic areas. However, despite this proposed functional split, it should be noted that modulation of addiction-related behaviors occurs in both regions (Vertes, 2006, Van den Oever et al., 2010). The orbital frontal cortex (OFC) is located ventrolaterally to the mPFC in rodents. The OFC is thought to be an important association area as it receives significant input from sensory areas and it has been shown to play a critical role in

determining outcome expectancies. In addition, its function is significantly altered by drug use (Öngür and Price, 2000, Schoenbaum et al., 2006).

Connectivity by region

In rats, the mPFC receives cholinergic inputs from the basal forebrain nuclei, noradrenergic inputs from the locus coeruleus (LC), serotoninergic inputs from the dorsal and median raphe nuclei, and dopaminergic inputs from the VTA and SNc (Heidbreder and Groenewegen, 2003, Groenewegen and Uylings, 2000, Uylings et al., 2003). The mPFC forms reciprocal projections with all of these areas (Heidbreder and Groenewegen, 2003, Van den Oever et al., 2010, Thierry et al., 2000, Carr and Sesack, 2000, Uylings et al., 2003, Zaborszky et al., 1997, Gaykema et al., 1991, Jodo et al., 1998). Although both the dmPFC and vmPFC receive inputs from LC, dorsal raphe, VTA/SNc, and glutamatergic inputs from several cortical and thalamic sources, there are also key differences in their connectivity patterns with these regions. The dmPFC projects to motor cortex, brainstem, thalamus, tectum, amygdala, dorsal striatum and the nucleus accumbens (NAc) (Vertes, 2006, Hoover and Vertes, 2007) whereas the vmPFC projects to midline thalamic nuclei, brainstem, bed nucleus of stria terminalis, hypothalamus, amygdala, and NAc (Vertes, 2006, Hoover and Vertes, 2007). In addition, the vmPFC is more likely to receive input from midline thalamic and 'limbic' cortices, such as hippocampus, orbital cortex, and PFC compared to the dmPFC (Vertes, 2006, Hoover and Vertes, 2007). The inputs from hippocampus originate in the CA1 and subiculum regions and project throughout the vmPFC (Swanson, 1981, Jay et al., 1989, Jay and Witter, 1991, Thierry et al., 2000, Vertes, 2006). Additionally, projections from the hypothalamus and amygdala are more heavily concentrated in

vmPFC compared to dmPFC (Hoover and Vertes, 2007). Within the vmPFC, there is also some separation in connectivity, as prelimbic cortex projects to the BLA whereas infralimbic cortex projects to the intercalated cells (Mcdonald et al., 1996, Berretta et al., 2005, Peters et al., 2009). There also seems to be some topographical distinction in the cholinergic innervation of these regions as the lateral portion of the basal forebrain targets dmPFC while the medial portion targets vmPFC (Hoover and Vertes, 2007). Finally, the OFC forms strong reciprocal projections with the thalamus, amygdala, and subiculum as well as provides input to the VTA, substantia nigra, dorsal striatum and NAc (Groenewegen, 1988, Kita and Kitai, 1990, Groenewegen et al., 1991, Öngür and Price, 2000, Schoenbaum et al., 2006, Hoover and Vertes, 2011, Arguello et al., 2016, Gremel et al., 2016). In addition to these regions, the OFC has a number of intracortical projections as well as additional projections to the septum, substantia innominata, raphe, claustrum, and periaqueductal gray (Hoover and Vertes, 2011).

C. Striatum Composition

The striatum is the major integration site of the cortico-basal ganglia-thalamic circuit, and as such receives a large variety of inputs. In particular, it receives cholinergic inputs from striatal interneurons and brainstem sources (e.g. laterodorsal tegmental area and the pedunculopontine nuclei) and GABAergic inputs from striatal interneurons (Kita, 1993, Dautan et al., 2014). Additionally, it receives dopaminergic inputs from the ventral tegmental area (VTA) and the substantia nigra (SNr) and glutamatergic inputs from several areas, including cortex, hippocampus, amygdala, and thalamus (Swanson, 1982, Phillipson and Griffiths, 1985, Finch, 1996, Groenewegen et al., 1999, Britt et al., 2012). These glutamatergic inputs make contact on the heads of

dendritic spines of the striatal GABAergic medium spiny projection neurons (MSNs) whereas dopaminergic inputs synapse onto the spine neck, allowing for an important and complex interaction between these two inputs in modulation of MSN activity (Freund et al., 1984, Xu et al., 1989).

The striatum itself can be divided into two main regions, the dorsal striatum and the nucleus accumbens (NAc), and is comprised of multiple neuronal phenotypes including four different types of interneurons (i.e., cholinergic interneurons and GABAergic interneurons, which express either parvalbumin, calretinin, or nitric oxide synthase/neuropeptide Y/somatostatin) (Kemp and Powell, 1971). However, the majority of striatal neurons (~95%) are MSNs (Kemp and Powell, 1971). The striatal MSNs in the dorsal striatum can be subdivided into two classes based on their projection patterns, as well as their neuropeptide and receptor expression. MSNs that send monosynaptic projections to the basal ganglia output nuclei (i.e. the SNr and the globus pallidus internal (GPi)) and express dopamine D1 receptors along with the neuropeptides dynorphin and substance P, form part of the direct pathway (dMSNs). MSNs that indirectly project to basal ganglia output nuclei via the globus pallidus external (GPe) and the subthalamic nucleus (STN) and express dopamine D2 receptors and the neuropeptide enkephalin, form part of the indirect pathway (iMSNs) (Gerfen and Surmeier, 2011, Wall et al., 2013). However it should be noted that the MSN projections are not entirely segregated, as some dMSNs send axon collaterals to the GPe/ventral pallidum (VP) (Fujiyama et al., 2011).

Classically, these two striatal MSN populations are thought to have opposing effects on basal ganglia output. Activation of the dMSNs causes a net excitation of the

thalamus resulting in a positive cortical feedback loop; thereby acting as a ‘go’ signal to initiate behavior. Activation of the iMSNs, however, causes a net inhibition of thalamic activity resulting in a negative cortical feedback loop and therefore serves as a ‘brake’ to inhibit behavior (Gerfen et al., 1982, Albin et al., 1989, Deniau and Chevalier, 1992, Gerfen and Surmeier, 2011, Calabresi et al., 2014). Additionally, basal ganglia output can be influenced via the hyperdirect pathway, which is a monosynaptic excitatory projection from the cortex to the STN that results in SNr excitation upon activation (Kita et al., 1983). Adding to the complexity of this circuit, the SNr itself projects back to the striatum as well as to the cortex, providing dopaminergic feedback to these structures (Gerfen et al., 1987).

Although these two striatal output pathways also exist in the NAc, the efferent targets of the MSNs are distinct from those in the dorsal striatum and the pathway segregation is much less complete. Specifically, iMSNs in the NAc (i.e., those neurons that express dopamine D2 receptors) project to the VP whereas dMSNs in the NAc (i.e., those neurons that express dopamine D1 receptors) project primarily to the VTA and SNr but also send axon collaterals to the VP (Chang and Kitai, 1985, Lu et al., 1998, Zhou et al., 2003, Tripathi et al., 2010, for review see Smith et al., 2013). It should also be noted that there is a small population of neurons in the NAc that coexpress both D1 and D2 receptors, though this is largely restricted to the NAc shell (Bertran-Gonzalez et al., 2008). For the purpose of providing clarity, we have operationally defined dMSNs as neurons that express dopamine D1 receptors and primarily target the VTA and/or SNr whereas iMSNs are neurons that express dopamine D2 receptors and primarily target the VP or GPe. However, it is important to recognize that depending on the targeting

method for a particular manipulation that was performed and the region it was performed in, the distinction between the indirect and direct striatal pathways in a given study may be less than complete.

In addition to the anatomical distinctions described above, the NAc and the dorsal striatum also differ functionally (Haber and McFarland, 1999, Berke and Hyman, 2000, Haber, 2003, Pennartz et al., 2011). Specifically, the NAc, which is typically associated with limbic areas, regulates affective components of behavior, including motivational and emotional processes (Berke and Hyman, 2000, Meredith et al., 2008, Pennartz et al., 2011). Neurons in the NAc core and NAc shell subdivisions also differ functionally. The NAc core is involved in the processing of conditioned stimuli whereas the NAc shell is more important in the processing of unconditioned stimuli; these differences are thought to be associated with variations in the morphology (shell neurons are smaller and significantly less spiny) and projection patterns of these subregions (Heimer et al., 1997, Groenewegen et al., 1999, Meredith et al., 2008). In contrast, the dorsal striatum, which contains both dorsomedial and dorsolateral aspects, receives dense innervation from neocortical areas, including motor areas (Berke and Hyman, 2000). The dorsomedial striatum is important in the performance of goal-directed behaviors whereas the dorsolateral striatum regulates habitual behaviors (Everitt and Robbins, 2005, Pennartz et al., 2011). Nonetheless, the striatal sub-regions are not independent, as limbic and cortical information can be passed from the NAc shell, through the core, and to the dorsal striatum in an ascending spiral via midbrain dopamine neurons (Haber et al., 2000).

Both the NAc and the dorsal striatum receive dense innervation from midbrain dopamine neurons, and these inputs have long been implicated in addictive behaviors (Swanson, 1982, Robinson and Berridge, 1993, Berke and Hyman, 2000, Everitt and Robbins, 2005, Gerfen and Surmeier, 2011). In particular, the primary pharmacological effects of psychostimulants are on dopamine release and reuptake mechanisms and while not their primary site of action, all other classes of abused drugs also increase striatal dopamine levels (Di Chiara and Imperato, 1988, White and Kalivas, 1998, Willuhn et al., 2010, Vander Weele et al., 2014). Since dMSNs express excitatory, Gs/olf-coupled dopamine D1 receptors and iMSNs express higher affinity, inhibitory Gi/o-coupled dopamine D2 receptors, drug-induced dopamine release has opposite effects on these two classes of MSNs. In addition, dopamine can have profoundly different effects on the plasticity of cortical inputs to these two striatal cell populations (Gerfen and Surmeier, 2011, Baik, 2013). For example, rewarding events (e.g. drug-taking) lead to dopamine release in the striatum, which promotes long-term potentiation (LTP) in cortical synapses onto dMSNs while simultaneously producing long-term depression (LTD) in iMSNs. Conversely, during non-rewarding events, dopamine neurons pause, resulting in decreased dopamine release in the striatum, which increases the strength of cortical synapses onto iMSNs and reduces the strength of cortical synapses onto dMSNs (Reynolds et al., 2001, Tang et al., 2001, Calabresi et al., 2007, Kreitzer and Malenka, 2007, Cohen and Frank, 2009, Gerfen and Surmeier, 2011, Hong and Hikosaka, 2011).

D. Cortical inputs and outputs

Inputs to striatum

The PFC sends a dense glutamatergic projection to the striatum with individual cortical neurons having the capacity to innervate many medium spiny neurons (MSNs). These corticostriatal projections are topographically organized such that the most dorsal aspects (i.e., the anterior cingulate) project more strongly to dorsal striatum and the most ventral aspects (i.e., the infralimbic and ventral prelimbic regions) project primarily to the NAc shell (McGeorge and Faull, 1989, Vertes, 2006, Van den Oever et al., 2010). Prelimbic cortex also projects to the NAc core (Peters et al., 2009).

mPFC to NAc: The mPFC has a diverse array of downstream targets; however, the mPFC to NAc projection is by far the most investigated cortical circuit in addiction-related behaviors using viral targeting methods. The striatum plays a critical role in cocaine-induced behaviors (Everitt and Robbins, 2005, Berke and Hyman, 2000, Robinson and Berridge, 2000) and the mPFC provides a dense glutamatergic input to the striatum (McGeorge and Faull, 1989, Groenewegen et al., 1991, Van den Oever et al., 2010, Britt et al., 2012, Wall et al., 2013). Using channelrhodopsin to activate this mPFC input to the NAc core/shell, it was found that stimulation produced both a CPP to the stimulation-paired side and supported self-stimulation. However, these results were also seen when either hippocampal or amygdala inputs to the NAc core/shell were stimulated, indicating that the enhanced reward may be due to increased excitation irrespective of the source pathway (Britt et al., 2012). As well, another study was unable to achieve self-stimulation of the mPFC to NAc core/shell pathway, although the discrepancies between these studies have been attributed to the differences in optogenetic stimulation parameters (Stuber et al., 2011). The effects of non-contingent cocaine experience on plasticity of mPFC neurons projecting to the NAc core has been

examined using optically-evoked excitatory postsynaptic currents (EPSCs) via channelrhodopsin. It was found that cocaine exposure increased *N*-methyl-d-aspartate receptor (NMDAR) function at mPFC to dopamine D1 receptor (D1R)-expressing MSN synapses in the NAc core, but not at non-D1R-expressing MSN synapses (Joffe and Grueter, 2016). Finally, the role of mPFC projections to the NAc core have also been examined in patterns of alcohol use that are associated with altered NMDAR functioning at mPFC to NAc synapses. Using halorhodopsin-induced optogenetic inactivation of these projections, a significant reduction in aversion-resistant alcohol intake was observed (Seif et al., 2013).

dmPFC to NAc: Cortical inputs from the dmPFC into the NAc core/shell have also been examined in addiction-related behaviors using a combinatorial chemogenetic targeting approach (i.e., expressing a Cre-recombinase (Cre)-dependent Gi/o-DREADD in the dmPFC and a retrograde canine adenovirusexpressing Cre (CAV-Cre) in the NAc). It was found that reducing dmPFC activity to the NAc core/shell transiently decreases amphetamine sensitization but has no effect on cocaine self-administration under a progressive ratio schedule of reinforcement (Kerstetter et al., 2016). In addition, prior chemogenetic inhibition of these neurons during cocaine-self-administration lead to an enhancement of drug-seeking on the first days of extinction as well as a paradoxical increase in cocaine-primed reinstatement of drug-seeking. However, chemogenetic inhibition of this dmPFC to NAc core/shell pathway during a reinstatement test attenuated drug-seeking to the cocaine prime (Kerstetter et al., 2016).

Similar findings on reinstatement have been reported when optogenetic inhibition by halorhodopsin was performed on prelimbic projections to the NAc core, as cue-

induced, cocaine-primed, and cue + cocaine- primed reinstatement of cocaine-seeking were all reduced (Stefanik et al., 2016, Stefanik et al., 2013). This optogenetic inhibition of prelimbic inputs to the NAc core also decreased the spine head diameter, AMPA/NMDA ratio, and spontaneous EPSC amplitude in NAc core MSNs produced by cue-induced reinstatement (Stefanik et al., 2016). Similarly, withdrawal from cocaine self-administration produces maturation of silent synapses in the prelimbic to NAc core pathway, and optogenetically reversing this process using channelrhodopsin and an LTD stimulation protocol inhibited incubation of cocaine craving (Ma et al., 2014).

vmPFC to NAc: The role of the vmPFC projections to the Nac core/shell have been examined more extensively than the dmPFC projections, and there are several optogenetic studies indicating that glutamatergic signaling from this pathway is enhanced following cocaine exposure, and regulates its behavioral effects. For instance, optogenetically-induced LTD on infralimbic terminals in the NAc core/shell (via channelrhodopsin) was sufficient to reverse the cocaine-induced potentiation of glutamatergic neurotransmission on D1R-expressing MSNs caused by non-contingent cocaine administration, as well as to block the expression and persistence of cocaine-induced locomotor sensitization (Pascoli et al., 2012). In addition, optical stimulation of infralimbic terminals within the NAc shell (via channelrhodopsin) revealed that withdrawal from cocaine self-administration increases glutamatergic signaling in D1R-expressing MSNs but not dopamine D2 receptor (D2R)-expressing MSNs (Pascoli et al., 2014). However, although optogenetically-induced LTD of infralimbic terminals on these D1R neurons prior to cue-induced reinstatement was sufficient to restore normal transmission, it increased lever responding non-discriminately (i.e., on both the cocaine-

associated and non-associated levers (Pascoli et al., 2014). Optogenetic stimulation via channelrhodopsin has also been used to demonstrate that the presynaptic release probability at vmPFC to NAc shell synapses is enhanced following cocaine withdrawal (Suska et al., 2013). This withdrawal from cocaine produces maturation of silent synapses in the infralimbic to NAc shell pathway, and optogenetically reversing this process (via channelrhodopsin and an LTD stimulation protocol) enhances craving (Ma et al., 2014). Finally, chemogenetic stimulation of the vmPFC to NAc shell pathway (via a Cre-dependent Gq-DREADD combined with CAV-Cre) significantly inhibited cue-induced, but not drug-primed, reinstatement of cocaine-seeking, but only after extinction training (i.e., there was no effect after abstinence) (Augur et al., 2016). Taken together, these studies suggest that cocaine produces a potentiation in the vmPFC to NAc pathway, and inhibition of this pathway can reduce both cocaine-induced neural plasticity as well as addiction-related behaviors. Nonetheless, as some findings do not fully support this idea, this work also highlights the fact that subregions within both the vmPFC and the NAc appear to play distinct roles in regulating the effects of cocaine.

OFC to dorsal striatum: While most of the viral studies tracing the role of cortical inputs to striatum in addiction behaviors have focused on the role of the mPFC to NAc projection, there has also been some intriguing, related work conducted on the projections from the OFC to dorsal striatum. Using a retrograde HSV-Cre virus in dorsal striatum and a Cre-dependent Gi/o-DREADD in OFC, it was found that chemogenetic inhibition of OFC projections to dorsal striatum abolished goal-directed actions and left mice dependent on habitual strategies (Gremel et al., 2016). Further, elimination of the endocannabinoid CB1 receptor from this OFC to dorsal striatum projection (via a

retrograde HSV-flippase virus targeted to the dorsal striatum and a Flp-dependent Cre virus targeted to the OFC of Cre-dependent CB1 receptor knockout mice) blocked the transition from goal-directed to habitual strategies (Gremel et al., 2016). Although this study did not directly study drug behaviors, the transition from goal-directed to habitual actions is a critical one in addiction (Everitt and Robbins, 2005), and this work highlights the importance of future research into the role of OFC to dorsal striatum projections in the transition to compulsive drug-taking.

Corticostriatal anatomy

Each striatal MSN receives roughly 5000 synapses from cortex (Kincaid et al., 1998). These cortical axons can travel up to 900 μM within the striatum, with synaptic boutons spaced about 10 μM apart (Kincaid et al., 1998, DiFiglia et al., 1978). This cortical innervation of striatum arises from layer V of cortex, and these corticostriatal neurons can project both ipsilaterally and contralaterally to striatum, as well as within the cortex itself. In addition to these corticostriatal neurons, layer V also contains intratelencephalic (IT) neurons, which project primarily within cortex, and pyramidal tract (PT) neurons, which can project ipsilaterally to the pontine nuclei, within the cortex itself, or to a number of other subcortical structures, including striatum, thalamus, pons, subthalamic nucleus, substantia nigra pars compacta (SNpc), and spinal cord (Wilson, 1987, Morishima and Kawaguchi, 2006, Hirai et al., 2012, Kim et al., 2015, Reiner et al., 2003, Reiner et al., 2010, Shepherd, 2013, Kubota et al., 2016).

Although anatomical evidence indicates that these two types of corticostriatal projection neurons may preferentially project to different types of striatal MSNs (i.e., striatal neurons that project directly to the SN versus those that project to the SN via the

globus pallidus external and subthalamic nucleus), there is electrophysiological evidence to suggest that functionally this separation does not exist (Wilson, 1987, Cowan and Wilson, 1994, Lévesque et al., 1996, Reiner et al., 2003, Reiner et al., 2010, Kress et al., 2013). Nonetheless, in addition to having different projection patterns, these two types of corticostriatal neuron also have morphological and electrophysiological differences. The PT-type neurons have a greater hyperpolarization activated current, exhibit a more tonic firing style, are larger, and have a more prominent apical dendrite (Morishima and Kawaguchi, 2006, Dembrow et al., 2010, Suter et al., 2013, Mason and Larkman, 1990, Hattox and Nelson, 2007, Miller et al., 2008, Gee et al., 2012, Shepherd, 2013). In addition, these two neuronal populations express different transcription factors (e.g. IT-type neurons express SATB2 and p11 whereas PT-type neurons express CTIP2 and FEZF2) and respond differently to dopamine, serotonin, and acetylcholine (Gaspar et al., 1995, Avesar and Gulledge, 2012, Dembrow et al., 2010, Shepherd, 2013).

VTA connections

Although the PFC projections to VTA have not been directly examined in drug-related behaviors, the available evidence on the anatomy of this circuit and the functional importance of both structures in addiction makes this pathway an appealing avenue of future research. Nonetheless, some viral targeting work has been done to examine connections between the mPFC and the VTA in motivated behaviors relevant to addiction. Specifically, removing NMDA receptors from the VTA and its inputs (using transgenic mice in conjunction with CAV-Cre) blocked Pavlovian learning, while restoring NMDA receptors to mPFC neurons projecting to the VTA (via an NR1 subunit-

encoding virus) was sufficient to restore Pavlovian learning (Parker et al., 2011). The VTA provides an important reciprocal pathway back to the PFC and salient environmental stimuli (e.g., a reward-associated cue) can induce burst firing of dopamine neurons in the VTA (Schultz, 2002, Fiorillo et al., 2003). Following optical stimulation of VTA dopamine terminals in the prelimbic cortex (using a Cre-dependent channelrhodopsin and transgenic rats that express Cre under the tyrosine hydroxylase (TH) promoter), it was found that mimicking this burst pattern of firing decreased intrinsic inhibition in pyramidal neurons in layer V of prelimbic cortex (Buchta et al., 2017). Similar results were obtained following more sustained chemogenetic activation, using either a Cre-dependent Gq-DREADD or Gs-DREADD (Buchta et al., 2017). These results are consistent with the idea that increased dopamine release from the VTA enhances mPFC neuronal activity to drive cocaine-seeking behavior (Goldstein and Volkow, 2011, Kalivas, 2009, McFarland and Kalivas, 2001). In contrast, Kabanova et al. (2015) found that channelrhodopsin-mediated activation of VTA neurons projecting to the mPFC produces excitatory postsynaptic potentials in cortical interneurons but inhibitory postsynaptic potentials in layer V pyramidal projection neurons. These effects were blocked by glutamate receptor antagonists, suggesting that they are mediated by glutamatergic drive from the VTA (Kabanova et al., 2015). The seemingly disparate results of these two studies could be due to variance in a number of experimental parameters, including the species used, the region of mPFC targeted, the subtype of VTA neurons expressing the transgenes, and the optical stimulation protocol. These last two variables, in particular, are likely to result in very different levels of released dopamine and/or glutamate from the VTA following

stimulation, which could have distinct effects on mPFC neuronal activity. Finally, these studies did not identify which subtype of pyramidal neurons (i.e., IT or PT) they were recording from. Given that these two populations respond differentially to dopamine (Gaspar et al., 1995, Shepherd, 2013), it is possible that the results were reflective of only one of the populations. This raises the intriguing possibility that cue-induced dopamine release could have differential effects on IT and PT neurons, and perhaps it is the relative balance in activity of these two neuronal populations that drive drug-seeking behavior.

Table 1. Summary of major viral tools used in this thesis.

Viral Tool	Function
Channelrhodopsin (ChR2)	Optical activation produces influx of sodium ions leading to increased neuronal firing
EnvA-ΔG-rabies-GFP	A modified monosynaptic rabies virus that allows for single synapse retrograde tracing when used in conjunction with TVA and RG helper viruses
TVA	Avian receptor that allows the modified EnvA G-deleted rabies to enter mammalian cells
RG	Rabies glycoprotein B19G which allows for the monosynaptic retrograde travel of the EnvA G-deleted rabies
$G_{i/o}$-DREADD	Ligand-driven activation increases $G_{i/o}$ signaling pathways leading to reduced neuronal firing
CAV-Cre	A canine adenovirus vector that allows for retrograde transport of Cre recombinase (Cre-)
HSV-Cre/HSV-Flp	A herpes simplex virus 1 vector that allows for retrograde transport of Cre- (or Flippase)
hSyn	Strong promoter that is specific to neurons
CAG/CMV	General promoters that drive strong expression
GCaMP	Intracellular calcium binds to the GCaMP protein resulting in fluorescence

E. Thesis Overview

The overarching goal of the work described in this thesis is to dissect and explore the different psychological components that underlie drug addiction through a variety of behavioral models and to investigate the role of corticostriatal circuits in these various components. In Chapter 2, I will analyze an intermittent access self-administration model and its validity as a model for individual differences in addiction susceptibility as well as its effects on intracellular calcium signaling in the dorsomedial striatum. Chapter 3 describes the discovery of distinct striatal-projecting circuits in the ACC involved in the positive and negative components of drug experience. Specifically, we find that IT neurons participate to the negative aspects of cocaine use and PT neurons contribute to its reinforcing properties. Chapter 4 builds on chapter 3 to examine if PT neurons, given their role in the reinforcing properties of cocaine, can also modulate drug-seeking and the motivation to take drug. Chapter 5 provides a discussion of this work and some future directions.

Chapter 2

Modeling individual differences in drug addiction with intermittent access self-administration

*This chapter is currently in preparation as an article for publication with Isah G. Webb, Lindsay M. Yager, and Susan M. Ferguson as co-authors.

AFG, IGW, and LMY performed the behavioral experiments. AFG performed GCaMP imaging and data analysis. AFG and SMF designed the experiments and wrote the manuscript.

A. Abstract

Drug addiction is a chronic disease with a complex constellation of symptoms and characteristics. Many models have been discovered to study individual aspects of drug addiction; however, there is great translational value in finding an animal model that encompasses as many characteristics of human addiction as possible. In this chapter, we describe an intermittent access model of self-administration that produces a subset of animals that display many of the hallmarks of addiction. The addiction susceptible animals show escalation of intake, increased drug-seeking on cue-induced reinstatement, locomotor sensitization, and altered patterns of drug-seeking and consumption. Finally, we show that temporal pattern of self-administration can alter intracellular calcium signaling in the dorsomedial striatum.

B. Introduction

Drug addiction is a chronic, relapsing disease with high costs to both individuals and society through its effects on quality of life, productivity, healthcare and crime (Cartwright 2008; Leshner 1997; Robbins and Everitt, 1999). Although drug use is common, only a relatively small number of individuals who try drugs ever become addicts (Anthony et al., 1994; Penberthy et al., 2010; Grant and Dawson, 1998). Nonetheless, it is not well-understood why some people transition to drug addiction and others do not. Determining the neural correlates of addiction will help us to address this critical issue; however, it is necessary to first be able to effectively and accurately model drug addiction in animals. Yet this is not a trivial task, as addiction is composed of a constellation of symptoms, including compulsive drug-taking and -seeking, loss of control over drug intake and a persistent craving for the drug.

Over the years, a range of models have been put forth in an attempt to capture key features of addiction. For example, non-contingent models such as locomotor sensitization are useful for modeling the long-lasting effects of drugs associated with addiction, including sensitization of the neural circuits thought to underlie salience attribution (Robinson and Becker, 1986; Paulson et al., 1991; Robinson and Berridge, 1993). In addition, extended access drug self-administration models produce an escalation of drug intake that is translationally appealing, as it is one of the hallmark characteristics that separates recreational users from addicts (Ahmed and Koob, 1998), and prolonged access self-administration paradigms, such as the three-criteria model, are effective at capturing the individual differences seen following drug use (Anthony et al., 1994; Penberthy et al., 2010; Grant and Dawson, 1998; Deroche-Gamonet et al.,

2004). Of note, self-administration paradigms that report escalation of drug intake have consistently found a lack of locomotor sensitization (Ben-Sharar et al., 2004; Ahmed and Cador, 2006; Knackstedt and Kalivas, 2007; but see Ferrario et al., 2005), suggesting that escalation reflects a tolerance rather than a sensitization of the neural circuits associated with addiction. These self-administration paradigms also have considerable drawbacks associated with their long-lasting nature, including catheter patency considerations as well as the time- and resource-intensiveness of the experiments.

Intermittent access self-administration paradigms, in which animals go through alternating periods of drug access and no access throughout each session (Zimmer et al., 2012) are intriguing as they are thought to more readily mimic the pattern of drug-taking seen in humans (Beveridge et al., 2012). Interestingly, these models have been shown to produce higher motivation to take drug compared to continuous access models, and they produce sensitization as opposed to tolerance at the dopamine transporter (Zimmer et al., 2012; Calipari et al., 2013). Here, we describe a version of the intermittent access self-administration model that is shorter in both session length and total experiment length than the extended access and three-criteria models, but captures key features of addiction in a subset of individuals including escalation of drug intake, locomotor sensitization, and high levels of drug-seeking. In addition, we examined baseline changes in calcium signaling in dorsal striatum using 2-photon calcium imaging in an attempt to identify a neural correlate underlying the observed addict-like behavior.

C. Materials and Methods

Animals

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Use and Care Committee and adhered to NIH guidelines. Male Sprague-Dawley rats (n = 89 Envigo) weighing 250-274g upon arrival were single-housed in a temperature- and humidity-controlled vivarium on a 12 h light/dark cycle. Food was available *ad libitum* except during fixed ratio (FR) training when animals were mildly food restricted and given 20g chow per day. Water was available *ad libitum*. All procedures and experiments took place during the light cycle.

Drugs

Cocaine hydrochloride was obtained from the National Institute of Drug Abuse and dissolved in 0.9% sterile saline.

Viral Vectors

A GCaMP6m viral vector driven by the human synapsin promoter (AAV5-hSyn-GCaMP6m-WPRE-SV40) and packaged in adenoassociated virus serotype 5 (AAV5) was obtained from the University of Pennsylvania viral vector core with a titer of ~1 x 10^9.

Surgical Techniques

Rats were anesthetized with isoflurane (2-4%, inhalation, Patterson Veterinary) for all surgical procedures. Rats received an injection of meloxicam (0.2 mg/kg SC, Patterson Veterinary) prior to surgery for analgesia. Rats underwent post-operative monitoring for 3 days following each surgical procedure.

Rats were implanted with chronic indwelling catheters placed into the right jugular vein and attached to a back-mounted port as previously described (Crombag et al., 2000). Catheters were flushed daily with 0.2 mL of gentamicin (5 mg/mL, Patterson Veterinary) dissolved in sterile saline to prevent occlusions and infections. Rats received injections of 0.2 mL sodium brevital dissolved in sterile saline (10 mg/mL IV, Patterson Veterinary) to determine catheter patency prior to the first session, and after the last session, of self-administration. Catheters were considered patent if rats became ataxic within 5 seconds of the infusion.

For calcium imaging experiments, one week prior to catheter surgery, animals received viral infusions of hSyn-GCaMP6m obtained from the University of Pennsylvania viral vector core using standard stereotaxic procedures. Briefly, 27-gauge stainless steel needles attached to gas-tight syringes (Hamilton Company) were used to infuse hSyn-GCaMP6m (1.0 µL) into the dorsomedial striatum (DMS; coordinates relative to bregma and from skull surface, A/P: + 0.2 mm, M/L: ± 2.0 mm, D/V: - 4.1 mm) at a rate of 0.4 µL/min. Needles were left in place for an additional 5 min infusion to allow for diffusion away from the infusion site.

Cocaine Self-Administration

Self-Administration Chambers

Self-administration occurred in standard operant chambers (Med Associates) equipped with two retractable levers, two white stimulus lights (one located above each lever), a white house light located on the back of the chamber, a house fan, and metal grid floor. A syringe pump located outside of the box delivered cocaine via tubing

attached to a suspended swivel and the catheter backport, allowing for free movement of the rats.

Cocaine Self-Administration Procedure

At least 5 days after surgery, rats were trained to lever press for cocaine on an FR1 schedule for 5 days. Each training session began with the insertion of the two retractable levers. A lever press on the active lever resulted in a cocaine infusion (0.4 mg/kg/inf in 50 µL over 2.8s) and illumination of the white stimulus light above the active lever (4s). Additional presses on the active lever during the cue light presentation were recorded, but did not result in additional infusions. Pressing on the inactive lever had no programmed consequences (i.e., no cocaine and no light). The location of the active lever was counterbalanced across animals. The session ended after 3h or after the rat had received 10 infusions, whichever came first. Following completion of 5 days of FR1 training, rats were given 14 sessions (one session per day for 14 days) of either intermittent (n = 53) or continuous (n = 28) access to cocaine on a FR1 schedule of reinforcement. A single intermittent access session consisted of 5 min access to cocaine followed by a 25 min time-out period where the levers were retracted and cocaine was not available This cycle repeated for a total of 155 minutes resulting in 6 drug-available periods separated by 5 drug-unavailable periods. For continuous access, rats had access to the levers and cocaine for the entire 155 minutes of each session.

Extinction

A subset of intermittent and continuous access rats (n = 21) underwent extinction training at the conclusion of the self-administration sessions. Rats were put into the operant chambers for 60 minutes per session, and responses on the active and inactive

levers had no programmed consequences (i.e., no drug infusion or illumination of cue light). Rats underwent extinction training until they made fewer than 30% of the active lever responses that they had made on their first day of extinction for two consecutive days, with a minimum of 10 total extinction sessions.

Cue-induced Reinstatement

After rats had achieved the extinction criteria, they underwent a 60-minute cue-induced reinstatement test of cocaine-seeking. Rats were put into the operant chamber, and the session began with the extension of the levers and the illumination of the cue light above the active lever for 4 sec. Each active lever press resulted in a 4 sec presentation of the cue-light, but no drug infusion.

Locomotor Sensitization

A subset of intermittent and continuous access rats (n = 38) underwent testing to assess for the development of locomotor sensitization. Prior to the start of self-administration, rats underwent an initial locomotor test where they were placed into locomotor activity boxes (San Diego Instruments) and allowed to habituate for 30 minutes. The rats then received an injection of saline (0.9%, *ip*), followed 30 minutes later by an injection of cocaine (10 mg/kg, *ip*) and behavior was recorded for an additional 60 minutes. Twenty-four hours later rats began FR1 training followed by either 14 sessions of intermittent (n = 27) or continuous (n = 11) access self-administration. Two weeks after the last self-administration session, rats underwent a second locomotor test in order to assess locomotor sensitization (Fig. 2.6A).

Continuous Access Self-Administration following Intermittent Access Self-Administration

A subset of intermittent access rats (n = 8) were switched to the continuous access paradigm for 7 sessions following the end of the 14 sessions of intermittent access to determine if animals with a history of intermittent access would have an altered drug intake pattern when provided with increased opportunity and access to cocaine.

Calcium Imaging

Slice Preparation

Naive animals (n = 8) or animals that had undergone intermittent or continuous access self-administration (n = 18) were anesthetized and perfused with an ice cold, high-sucrose artificial cerebrospinal fluid (aCSF) solution (in mM: 210 sucrose, 5 KCl, 1.25$NaH_2PO_4 \cdot H_2O$, 3.5$MgSO_4 \cdot 7H_2O$, 0.5$CaCl_2 \cdot 2H_2O$, 26 $NaHCO_3$ and 10 D-glucose, osmolarity ~300 mOsm) 7-10 days after the end of self-administration. Rats were decapitated and their brains quickly removed and transferred to a chamber of cold, high-sucrose aCSF. Three hundred micron thick coronal slices were taken with a vibrating microtome, while the brains were submerged in cold, high-sucrose aCSF. Slices were then transferred to a chamber filled with *N*-methyl-D-glucamine protective recovery aCSF (in mM: 93 *N*-methyl-D-glucamine, 93 HCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10$MgSO_4 \cdot 7H_2O$ and 0.5$CaCl_2 \cdot 2H_2O$, osmolarity ~300 mOsm) for less than 15 minutes. Finally, slices were transferred to a chamber filled with room temperature aCSF (in mM: 119 NaCl, 5 KCl, 1.3$MgSO_4 \cdot 7H_2O$, 2.5$CaCl_2 \cdot 2H_2O$, 1$NaH_2PO_4 \cdot H_2O$, 16.2 $NaHCO_3$, 11 D-glucose and 10 HEPES, osmolarity ~300 mOsm) and allowed to rest for ~1 hour. All solutions were bubbled with a 95 percent O_2–5 percent CO_2 mixture.

Image Acquisition and Processing

Slices were submerged in oxygenated aCSF (32°C), which was superfused throughout the experiments. Fluorescence images were captured at 5 Hz with an Olympus FV1000 upright microscope equipped with 25× 1.05 NA Ultra-objectives driven by a femtosecond pulsed MaiTai DeepSee laser (Spectra Physics) tuned to 890-nm excitation. After acquisition, images were processed using FIJI and FluoroSNNAP (Patel et al., 2015). ROIs were identified from image stacks, which were motion corrected. The Δ*F*/*F* record was calculated by subtracting raw fluorescence values with the mean of the lower 50 percent of previous 10-second values and dividing by the mean of the lower 50 percent of previous 10-second values (Patel et al., 2015). Calcium events were detected using a deconvolution method with a detection threshold of 3 standard deviations above the baseline.

Statistical Analysis

GraphPad Prism 7 was used for all statistical analyses. Unpaired t-tests were used to analyze differences in extinction responding (on day 1), reinstatement responding, and total intake. Linear regressions were used to analyze active presses across days and separate animals into escalators and non-escalators. Days 1-3 were excluded from this analysis due to instability in initial responding, which was likely the result of learning during the initial stages of the paradigm. Pearson correlations were used to analyze the relatedness of extinction responding (on day 1), reinstatement responding, and drug intake at the end of self-administration (average infusions over last 3 days). Repeated measures two-way ANOVAs were used to analyze group differences in infusions during continuous access following intermittent access, and

locomotor responding during habituation, following saline injection, and following cocaine injection. One-way ANOVAs were used to analyze group differences in calcium event rates, calcium event rise times, and calcium event fall times. For all comparisons, $\alpha \leq 0.05$. Data is graphed as mean ± SEM.

D. Results

Escalation in intermittent access self-administration as a marker of addiction severity

Active presses did not change over time with continuous access self-administration animals (one-way RM ANOVA, $F_{(1.469, 11.75)} = 1.346$, $p = 0.287$, Fig. 2.1A). However, active presses significantly increased over time in intermittent access animals (one-way RM ANOVA, $F_{(3.152, 66.19)} = 7.473$, $p = 0.0002$), indicating an escalation of intake over time. In order to determine if escalation of intake could be used as a marker for addiction severity in the intermittent paradigm, we examined individual animals to determine if all intermittent animals were exhibiting escalation or if only a subset of animals escalated. Linear regressions were run on active presses across sessions in intermittent access self-administration animals to distinguish between animals that showed significant escalation and those that did not. Intermittent escalators strongly increased their active presses across self-administration sessions (one-way RM ANOVA, $F_{(2.391, 26.3)} = 12.04$, $p < 0.0001$, Fig. 2.1C) while intermittent non-escalators did not (one-way RM ANOVA, $F_{(3.808, 34.27)} = 1.862$, $p = 0.142$, Fig. 2.1B). Due to the strong association between escalation of intake and addiction we hypothesized that escalation behavior would reliably predict performance on other addiction behaviors.

An altered pattern of drug consumption

We examined the inter-press intervals and the estimated cocaine concentrations achieved in order to investigate differences in the pattern of drug consumption and seeking. The estimated cocaine concentration curves generated for escalator and non-escalator intermittent access animals was not significantly different on session 1 (two-way RM ANOVA, no main effect of pattern $F_{(1,51)} = 0.459$, $p = 0.501$; main effect of time

$F_{(360,18360)}$ = 100.9, p < 0.0001; no effect of pattern x time interaction $F_{(360,18360)}$ = 0.354, p > 0.9999, Fig. 2.1D) or session 7 (two-way RM ANOVA, no main effect of pattern $F_{(1,51)}$ = 0.297, p = 0.588; main effect of time $F_{(360,18360)}$ = 168, p < 0.0001; no effect of pattern x time interaction $F_{(360,18360)}$ = 0.699, p > 0.9999, Fig. 2.1E), but was significantly different by session 14 of self-administration (two-way RM ANOVA, main effect of pattern $F_{(1,51)}$ = 4.501, p = 0.039; main effect of time $F_{(360,18360)}$ = 200.8, p < 0.0001; effect of pattern x time interaction $F_{(360,18360)}$ = 2.477, p < 0.0001, Fig. 2.1F). This effect was largely driven by elevated concentrations for the escalators during the latter half of the session. This is an interesting difference from escalation in the extended access model of self-administration, which produces escalation during the loading phase at the beginning of the session (Ahmed and Koob, 1998). Similarly, the inter-infusion intervals for intermittent escalators and non-escalators were not different during session 1 (two-way RM ANOVA, no main effect of pattern $F_{(1,51)}$ = 2.387, p = 0.129; main effect of time $F_{(19,969)}$ = 85.57, p < 0.0001; no effect of pattern x time interaction $F_{(19,969)}$ = 1.36, p = 0.138, Fig. 2.1G) or session 7 (two-way RM ANOVA, no main effect of pattern $F_{(1,51)}$ = 0.483, p = 0.49; main effect of time $F_{(19,969)}$ = 93.88, p < 0.0001; no effect of pattern x time interaction $F_{(19,969)}$ = 0.753, p = 0.764, Fig. 2.1H), but was significantly different by day 14 with escalators showing more “burst” pressing with more infusions with only 0-1 min intervals between them (two-way RM ANOVA, main effect of pattern $F_{(1,51)}$ = 4.255, p = 0.044; main effect of time $F_{(19,969)}$ = 175.4, p < 0.0001; effect of pattern x time interaction $F_{(19,969)}$ = 3.904, p < 0.0001, Fig. 2.1I). Continuous access animals also showed a pattern adaptation over time as their inter-infusion interval was significantly different on day 14 compared to day 1 (two-way RM ANOVA, main effect within session

$F_{(19,513)}$ = 54.68, p < 0.0001; main effect across session $F_{(1,27)}$ = 18.8, p = 0.0002; effect of within session x across session interaction $F_{(19,513)}$ = 19.24, p < 0.0001). Animals on continuous access also took significantly more drug over time compared to intermittent animals, likely due to their greater access (*t* test, t_{79} = 13.17, p < 0.0001, Fig. 2.4F).

Pattern of drug-seeking on a cue-induced reinstatement test

We tested the effect of self-administration pattern (intermittent access vs continuous access) on drug-seeking and relapse susceptibility by examining the number of active presses on a cue-induced reinstatement test. Intermittent escalators showed significantly more responses to a cue-induced reinstatement test compared to non-escalators (*t* test, t_{13} = 2.251, p = 0.042, Fig. 2.2B). During cue-induced reinstatement, intermittent escalators showed a pattern of increased "burst" pressing showing significantly higher presses with 0-1 min intervals between them (two-way RM ANOVA, no main effect of pattern $F_{(2,21)}$ = 3.336, p = 0.055; main effect of time $F_{(19,399)}$ = 122.7, p < 0.0001; effect of pattern x time interaction $F_{(38,399)}$ = 3.31, p < 0.0001, Fig. 2.2C).

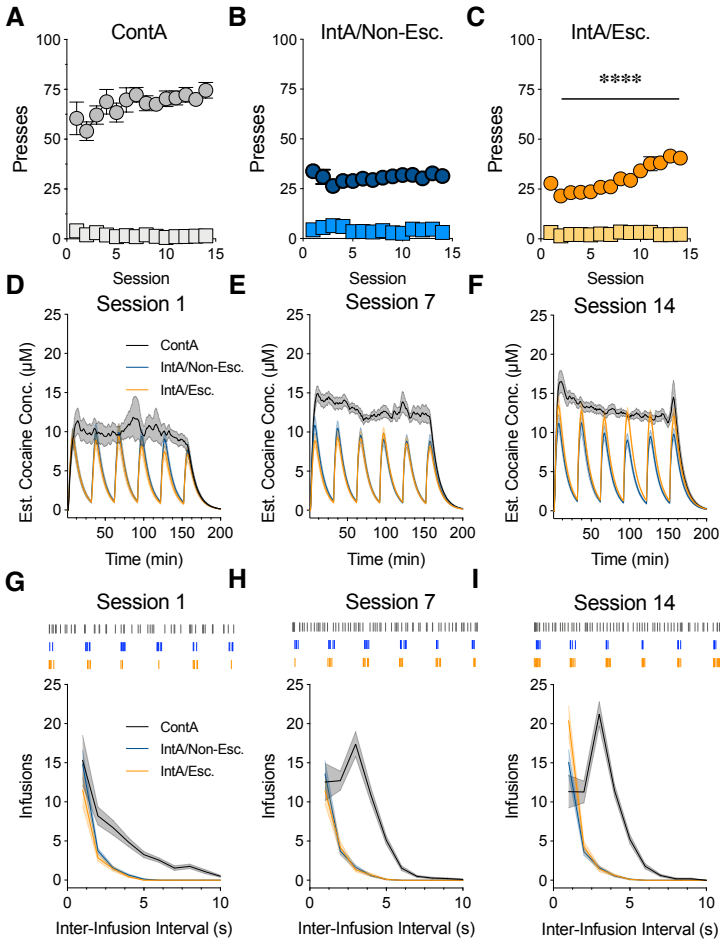

Figure 2.1. There is no difference between continuous and intermittent access self-administration on drug-seeking. (A) Active and inactive lever presses for continuous access self-administration. (B) Active and inactive lever presses for intermittent access non-escalators during self-administration. (C) Active and inactive lever presses for intermittent access escalators during self-administration. These animals significantly escalated their drug intake during intermittent access self-administration ($p < 0.0001$). (D) Estimated cocaine concentration on the first session of self-administration. Shaded portion represents SEM. (E) Estimated cocaine concentration on the seventh session of self-administration. Shaded portion represents SEM. (F) Estimated cocaine concentration on the fourteenth session of self-administration. Intermittent escalators reach significantly higher concentrations than non-escalators, especially in the second half of the session ($p < 0.0001$). Shaded portion represents SEM. (G) Inter-infusion interval on the first session of self-administration. Shaded portion represents SEM. Hash marks above the graph indicate

lever presses of representative animals. (H) Inter-infusion interval on the seventh session of self-administration. Shaded portion represents SEM. Hash marks above the graph indicate lever presses of representative animals. (I) Inter-infusion interval on the fourteenth session of self-administration. Intermittent escalators take significantly more infusions with short inter-infusion intervals compared to non-escalators ($p < 0.0001$). Shaded portion represents SEM. Hash marks above the graph indicate lever presses of representative animals.

Behavioral variability in addiction severity

Drug consumption, extinction, and reinstatement can all be used to examine different aspects of addiction. We tested if the two self-administration paradigms were producing coherent, consistent models of addiction by examining the relatedness of the three factors. Animals that had undergone continuous access self-administration showed no correlation between the amount of drug taken in the last 3 days of self-administration and the number of active presses on the first day of extinction (Pearson correlation, $r = 0.622$, $p = 0.074$, Fig. 2.2D) or the number of active presses on the cue-induced reinstatement test (Pearson correlation, $r = 0.393$, $p = 0.296$, Fig. 2.2E). There was also no correlation between the number of active presses on the first day of extinction and the number of active presses on the cue-induced reinstatement test (Pearson correlation, $r = 0.523$, $p = 0.148$, Fig. 2.2F). In contrast, animals that underwent intermittent access self-administration showed significant correlations between the amount of drug taken in the last 3 days of self-administration and the number of active presses on the first day of extinction (Pearson correlation, $r = 0.727$, $p = 0.001$, Fig. 2.2G) as well as the number of active presses on the cue-induced reinstatement test (Pearson correlation, $r = 0.711$, $p = 0.003$, Fig. 2.2H). The number of active presses on the first day of extinction and the number of active presses on the cue-induced reinstatement test were also strongly correlated (Pearson correlation, $r =$

0.734, p = 0.002, Fig. 2.2I). This correlational data provides evidence that the intermittent access self-administration paradigm produces a spread in behavior consistent with individual differences in addiction severity, which the continuous access model does not.

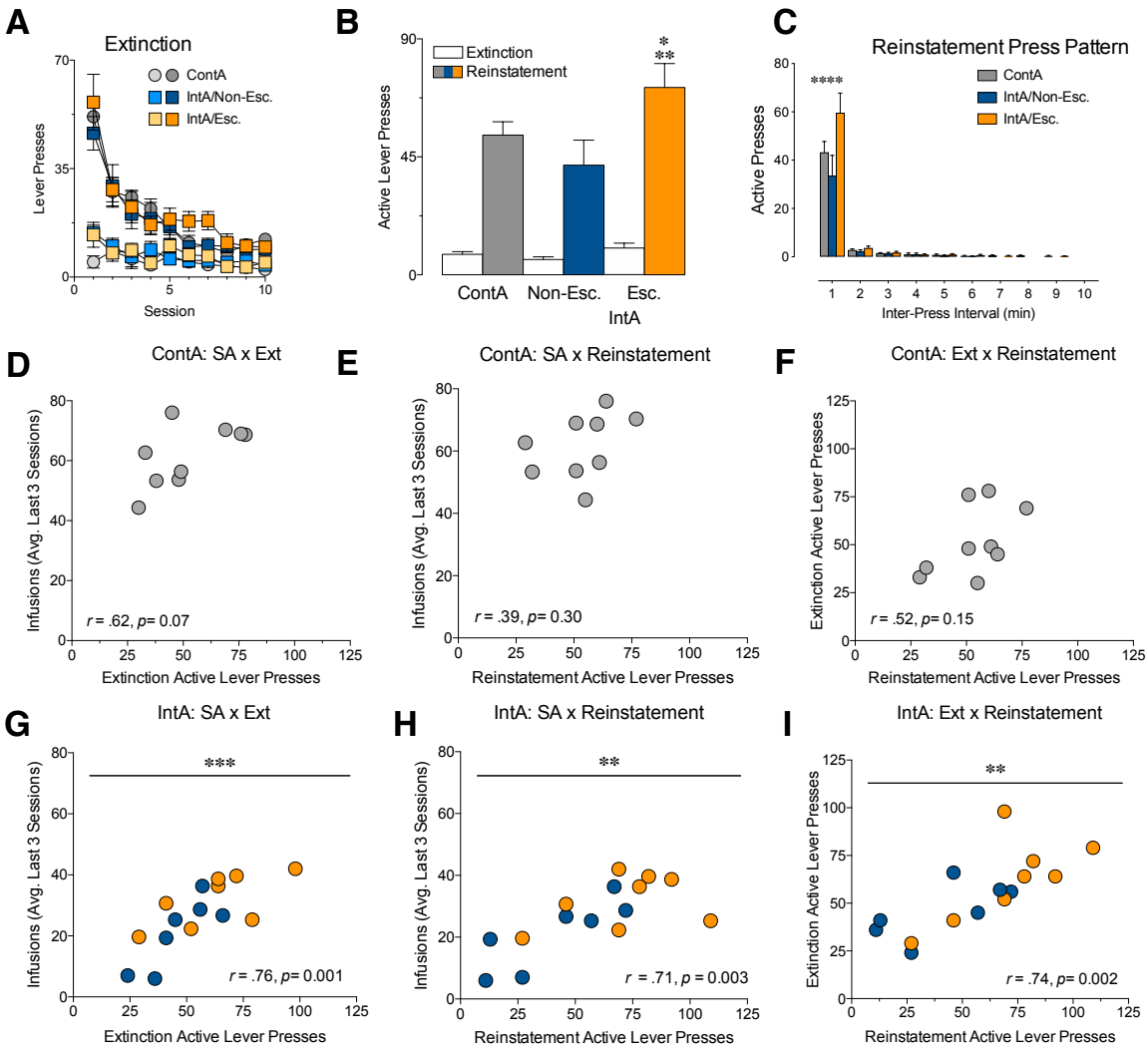

Figure 2.2. Intermittent access produces behavioral variability consistent with addiction susceptibility. (A) Active and inactive presses during extinction. (B) Intermittent escalators have significantly higher presses on a cue-induced reinstatement test compared to non-escalators and continuous animals (p = 0.042). (C) Intermittent escalators display a different pattern of seeking during a cue-induced reinstatement test with significantly more presses that have short inter-press intervals between them (p < 0.0001). (D) There is no correlation between drug intake at the end of self-

administration and drug-seeking on the first day of extinction for continuous access animals. (E) There is no correlation between drug intake at the end of self-administration and drug-seeking during cue-induced reinstatement for continuous access animals. (F) There is no correlation between drug-seeking on the first day of extinction and drug-seeking during a cue-induced reinstatement test for continuous access animals. (G) Higher drug intake at the end of self-administration is predictive of higher drug-seeking during the first day of extinction for intermittent access animals (p = 0.001). (H) Higher drug intake at the end of self-administration is predictive of higher drug-seeking during a cue-induced reinstatement test for intermittent access animals (p = 0.003). (I) Higher drug-seeking during the first day of extinction is predictive of higher drug-seeking during a cue-induced reinstatement test for intermittent access animals (p = 0.002).

Continuous access self-administration following intermittent access self-administration

Binge-taking behavior is considered a crucial aspect of addiction (Koob and Le Moal, 2001). Since intermittent access self-administration restricts the animals' access to drug to short, discrete periods, we examined if intermittent escalators and non-escalators would react differently when granted the increased drug access provided during continuous access self-administration. When switched to continuous access self-administration after intermittent access, escalators took more infusions on each day compared to the non-escalators, indicating that they may be more susceptible to engaging in binge-like behaviors when given the opportunity (two-way RM ANOVA, main effect of time $F_{(6,30)}$ = 2.948, p = 0.022; main effect of pattern $F_{(1,5)}$ = 6.956, p = 0.046; no effect of time x pattern interaction $F_{(6,30)}$ = 0.999, p = 0.445; Fig. 2.3C).

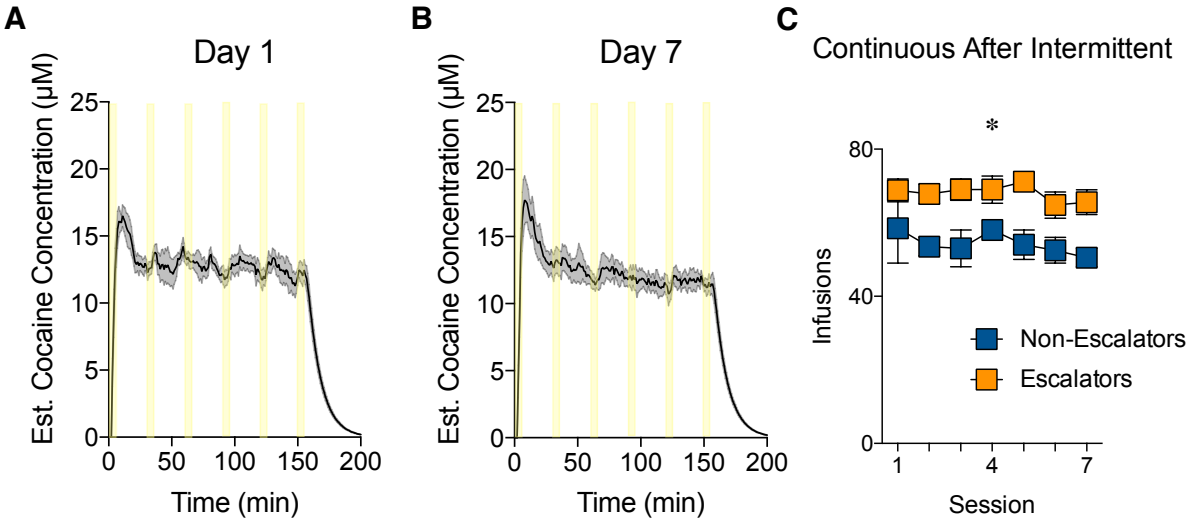

Figure 2.3. Continuous access self-administration following intermittent access self-administration. (A) Estimated cocaine concentration of the first day of continuous access self-administration following intermittent access self-administration. Shaded portion represents SEM. (B) Estimated cocaine concentration of the seventh day of continuous access self-administration following intermittent access self-administration. Shaded portion represents SEM. (C) Intermittent escalators take significantly more infusions of cocaine compared to non-escalators when put on a continuous access paradigm (p = 0.046).

Continuous versus intermittent access self-administration as a sensitizing paradigm

Although escalation of intake has been observed before with other self-administration models, it is rare for self-administration to induce both escalation and locomotor sensitization (Ben-Sharar et al., 2004; Ahmed and Cador, 2006; Knackstedt and Kalivas, 2007). We tested groups of continuous and intermittent access self-administration animals with an injection of cocaine before and two weeks after the self-administration to determine if either model could produce sensitization and if within the intermittent access model, escalation of intake would continue to act as a useful marker for addiction severity. Self-administration pattern did significantly affect locomotor sensitization with intermittent escalators showing the greatest difference (two-way RM

ANOVA, no main effect of pattern $F_{(2,35)}$ = 0.006, p = 0.994; main effect of time $F_{(1,35)}$ = 26.02, p < 0.0001; effect of pattern x time interaction $F_{(2,35)}$ = 4.063, p = 0.026, Fig. 2.6D). There were no group differences during habituation (two-way RM ANOVA, no main effect of pattern $F_{(2,35)}$ = 0.2569, p = 0.7749; main effect of time $F_{(1,35)}$ = 5.738, p = 0.022, no pattern x time interaction $F_{(2,35)}$ = 1.912, p = 0.163, Fig. 2.6B) or following a saline injection (two-way RM ANOVA, no main effect of pattern $F_{(2,35)}$ = 0.855, p = 0.434; main effect of time $F_{(1,35)}$ = 19.67, p < 0.0001, no pattern x time interaction $F_{(2,35)}$ = 0.7558, p = 0.4771, Fig. 2.6C). There was no difference in total intake between escalators and non-escalators, indicating that the behavioral differences seen during self-administration and sensitization were not the result of increased cocaine use (*t* test, t_{51} = 0.08152; p = 0.935, Fig. 2.6F).

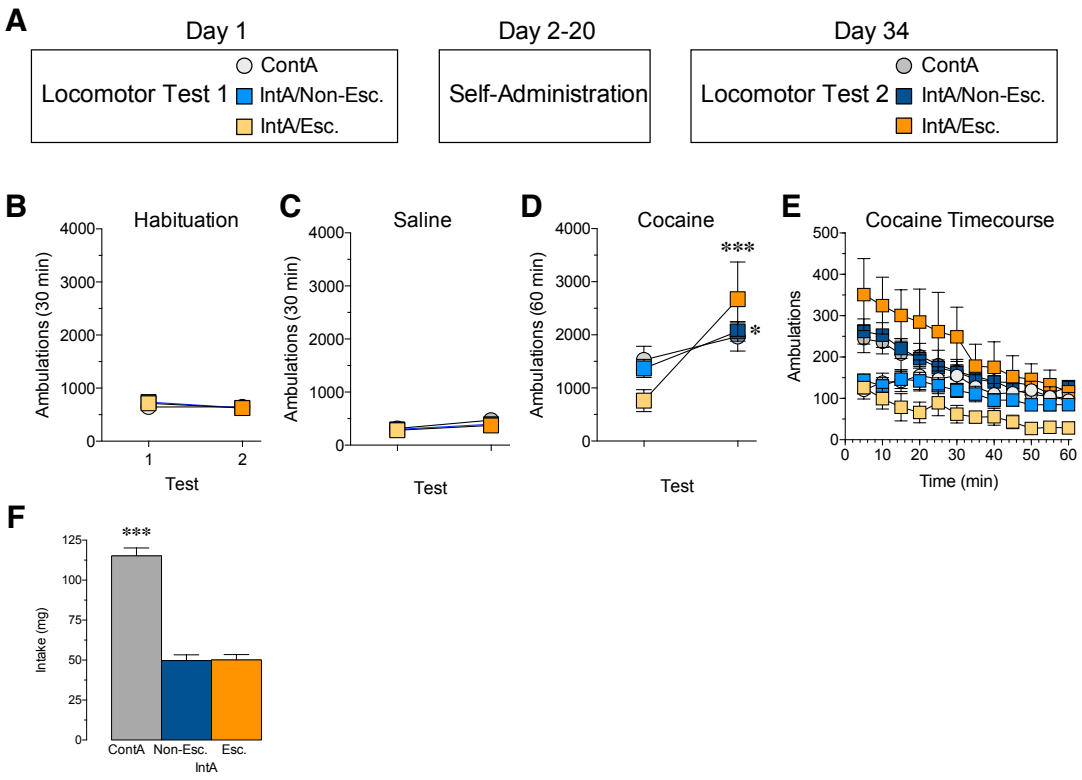

Figure 2.4. Intermittent escalators show sensitization to cocaine. (A) Animals underwent an initial locomotor test to determine their response to non-contingent cocaine, followed by FR training and either intermittent or continuous access self-administration. Two weeks after the completion of self-administration, animals underwent another locomotor test to determine if their locomotor response to cocaine had sensitized. (B) There were no group differences during the habituation phase. (C) There were no group differences during the saline phase. (D) Intermittent animals had undergone significant sensitization to cocaine with escalators showing the most robust sensitization (p = 0.026). (E) Locomotor responses during the initial and final locomotor tests. (F) Continuous access animals took the most drug during self-administration (p < 0.0001), but intermittent escalators and non-escalators did not differ in their total intake of cocaine over the course of self-administration.

Calcium events in DMS after self-administration

In order to determine how self-administration pattern affected calcium signaling in the DMS, we performed *ex vivo* 2-photon calcium imaging in DMS slices of animals 10-14 days after they completed either continuous intermittent access self-administration.

Animals that underwent continuous access self-administration had significantly more calcium events in DMS (one-way ANOVA, $F_{(3,104)}$ = 7.325, p = 0.0002, Fig. 2.7B). They also had significantly shorter rise and fall times compared to intermittent escalators (one-way ANOVA, $F_{(3,87)}$ = 3.162, p = 0.029; $F_{(3,87)}$ = 4.433, p = 0.006, Fig. 2.7C and 2.7D).

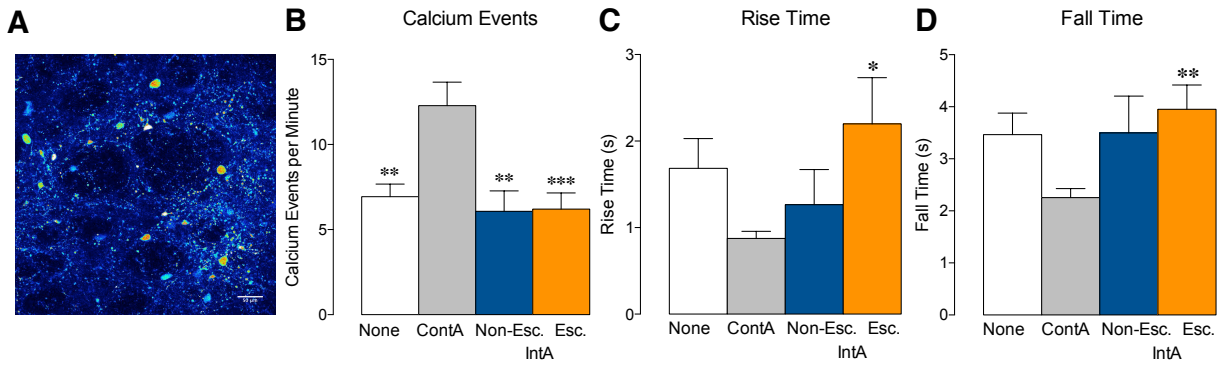

Figure 2.5. Temporal pattern of self-administration alters calcium signalling in DMS. (A) Expression of GCaMP6m in the dorsomedial striatum. (B) Continuous access animals had significantly more calcium events in DMS (p = 0.0002). (C) Continuous access animals had significantly shorter rise times of calcium events compared to intermittent escalators (p = 0.029). (D) Continuous access animals had significantly shorter fall times of calcium events compared to intermittent escalators (p = 0.006).

E. Discussion

Investigating individual differences in susceptibility to addiction is a major issue facing the addiction field going forward. We examined the ability of continuous access and a shortened intermittent access self-administration model to distinguish “addicted” animals from “non-addicted” animals. Intermittent access self-administration raises animals’ motivation to take drug compared to extended access models (Zimmer et al., 2012). We found that intermittent access self-administration produced a relevant spread of behavioral results that did not occur with the continuous access model. Drug intake at the end of self-administration, seeking during the first extinction session, and cue-induced seeking were all highly correlated in the intermittent paradigm with no correlation in the continuous paradigm. Although these data do not preclude the possibility that the continuous model of self-administration may be valuable to study individual components of drug addiction, they do imply that the continuous model is not as effective at producing a coherent, comprehensive model of addiction.

We found that the intermittent access paradigm produced an escalation of intake across sessions that did not occur with the continuous access model. Upon further investigation, we noticed that only ~40% of animals exposed to intermittent access showed escalation of intake while the remaining ~60% maintained stable intake across sessions. Escalation of intake over time is commonly considered a hallmark of addiction and we hypothesized that if the intermittent access paradigm was producing a meaningful spread in terms of addiction severity that escalation of intake could be a valuable grouping variable (Ahmed and Koob, 1998). We sought to assess the value of escalation in the intermittent access model as a marker for “addicted” animals by its

ability to predict behavioral outcomes on other critical components of addiction, such as susceptibility to relapse, sensitization, and pattern of drug-seeking and consumption. In addition to escalation, a chronic susceptibility to relapse is a crucial characteristic of addiction (Childress et al., 1999; McFarland and Kalivas, 2001). We found that animals that showed escalation of intake over time had higher seeking on a cue-induced reinstatement test.

Locomotor circuits are believed to overlap heavily with the neural circuits mediating drug craving and so locomotor sensitization is thought to represent the long-lasting changes in susceptibility to craving produced by drug addiction (Robinson and Berridge, 1993). It has been challenging, though, to find models that produce both escalation of intake as well as traditional locomotor sensitization (Ben-Sharar et al., 2004; Ahmed and Candor, 2006; Knackstedt and Kalivas, 2007). Intermittent access animals displaying escalation of intake also showed a robust locomotor sensitization compared to both the non-escalating animals and the continuous access animals. It should be noted that although the escalating animals showed an increase in consumption over time, the total amount of drug consumed during the full experimental paradigm by the escalators and non-escalators was not different, indicating that the behavioral differences we observed were not simply the byproduct of increased cocaine having a greater pharmacological effect. Our data builds on previous work showing that intermittent animals can sensitize within the self-administration session, by demonstrating that the locomotor sensitization persists for weeks after the end of self-administration, and that it is especially robust in the intermittent escalators (Allain and Samaha, 2018).

Interestingly, the seeking and consumption were not only enhanced for the escalators in the intermittent access model, but the pattern of seeking and consumption were significantly different compared to the non-escalators. We found that the animals that escalated their intake during the intermittent access model had higher pressing with low inter-press intervals during a cue-induced reinstatement test, perhaps indicating a lack of restraint as their pressing was composed of rapid, impulsive pressing. We noticed a similar difference in drug consumption between the escalators and non-escalators by the final self-administration session. The escalators had significantly more drug infusions with low-infusion intervals compared to the non-escalators, again demonstrating this impulsive, binge-like behavior. This is strengthened by our finding that when animals are put on a continuous access paradigm after completing intermittent access, the escalators take significantly more infusions compared to the non-escalators. This “binge” pattern of consumption is also common in human addicts (Gawin and Kleber, 1986). The differences in inter-infusion interval between the intermittent access animals and the continuous access animals is likely an effect of the paradigm design. The continuous access animals begin with a pattern not dissimilar from the intermittent access animals, but over time develop a pattern marked by a high level of presses with short intervals between them (loading phase) and a high level of presses with an intermediate interval between them (maintenance phase) while the parameters of the intermittent access paradigm prevent them from ever adopting the inter-infusion peak characteristic of maintenance behavior and instead forces them to undergo multiple loading phases per session.

The escalation behavior in the intermittent access paradigm was characterized by enhanced drug-intake during the second half of the self-administration session compared to the non-escalators. This is a departure from the escalation pattern seen with extended access models, which produce escalation at the beginning of the session (Ahmed and Koob, 1998). This data reflects the human clinical data on “binge” taking behavior well and could potentially be interpreted as a result of acute tolerance or acute sensitization. Human addicts typically engage in “binge” consumption behavior as they increase their drug-consumption in a single session in an attempt to counteract the rapid decrement of physical and psychological (“rush”) effects (Trinkoff et al., 1990; Brower et al., 1986). Although there is less clinical evidence for acute sensitization, it is possible that as the animals are exposed to the drug, their craving sensitizes and escalates as might be expected in cocaine-primed reinstatements. It is plausible that the escalators in the intermittent access paradigm are increasing their consumption within session due to an attempt to recapture the “rush” produced by the initial drug use in the session (acute tolerance) or due to a drug-induced ramping up of their craving systems (acute sensitization), or some combination of the two.

The dorsomedial striatum (DMS) is an important region for goal-directed actions, drug-seeking, and for the transition to drug addiction (Robbins & Everitt, 2002; Yin et al., 2005; Nonomura et al., 2018; Ito et al., 2002). We thought it likely that the behavioral differences in addiction severity produced by the intermittent access paradigm would be reflected by changes to the DMS. Intracellular calcium signaling in the striatum has also been implicated in both drug reward and sensitization (Barr et al., 2015; Mizuno et al., 2013; Yasui and Su, 2016). Surprisingly, we did not observe any differences in calcium

signaling in the DMS between escalators and non-escalators, although we did see many differences between calcium waves in the continuous access animals and the intermittent access escalators. We found that continuous access animals had more frequent, narrower calcium waves compared to intermittent escalators. Intracellular calcium signaling is a critical component of many signaling cascades and the broader effects of such a shift in the characteristics of these waves is unclear. The DMS is an important region in the transition to addiction, and so it is possible that we observed it too early or late in the transition to detect a difference between the escalators and non-escalators. It is also possible that changes may have been readily observed at a different time point after the end of self-administration. The changes that were observed between the continuous access and intermittent access escalators may have been driven by the significant differences in cocaine consumption as continuous access animals took the most cocaine of any group. There is evidence behaviorally that abstinence affects animals that have consumed more cocaine differently than those with lower consumption (Ahmed and Koob, 1998).

In conclusion, these results indicate that an intermittent access model of self-administration produces a meaningful variance in behavioral data that can be used to probe individual differences in addiction severity. A subset of animals in the intermittent access paradigm escalated their intake across sessions, had higher cue-induced drug seeking, locomotor sensitization, and binge-like behaviors compared to non-escalators. The paradigm could have significant translational and practical utility in future research.

Chapter 3

Dissociation of drug reward and aversion in striatal-projecting cortical cells

*This chapter is currently in preparation as a brief communication for publication with Isah G. Webb and Susan M. Ferguson as co-authors.

AFG and IGW performed the behavioral experiments. AFG performed anatomical tracing and data analysis. AFG and SMF designed the experiments and wrote the manuscript.

A. Abstract

Projections from prefrontal cortex to striatum play a critical role in drug addiction; however, these projections and their contribution to drug addiction have never been separated in a cell-type specific manner. We demonstrate that striatal-projecting intratelencephalic (IT) and pyramidal tract (PT) cortical neurons differentially contribute to the positive and negative components of drug experience. These findings suggest that there are distinct drug-reward and aversion circuits in the anterior cingulate cortex.

B. Brief Communication

Drug addiction is a prevalent and persistent disease marked biologically by molecular and synaptic adaptations in corticolimbic circuitry, and psychologically by a combination of positive and negative symptoms (Nestler 2001; Robbins & Everitt, 2002; Kalivas & O'Brien, 2008; Gay, 1982; Trinkoff et al., 1990). Accordingly, the abuse of drugs such as cocaine is driven by a complex interplay between the potent reinforcing effects of these drugs and the aversive states produced by extended and/or discontinued use, including stress and dysphoria (Gay 1982; Ettenburg and Geist, 1991; Washton and Gold, 1984; Anthony et al., 1989; Trinkoff et al., 1990; Wood et al., 2014). The anterior cingulate cortex (ACC) is an understudied, but intriguing, target for the dysregulated drug use characteristic of addiction as it responds to both positively- and negatively-valenced stimuli. For instance, chronic pain and remote fear memories, as well as sexual mating, cocaine and cocaine-associated cues all robustly activate the ACC (Johansen and Fields, 2004; Navratilova et al., 2015; Gao et al., 2017; Frankland et al., 2004; Frohmader et al., 2010; McLaughlin & See, 2003; Zahm et al., 2010; Neisewander et al., 2000; Zhou et al., 2014). In addition, the ACC plays an important role in decision-making, especially in tasks that require an evaluation of rewards and their associated costs (Walton et al., 2003; Schweimer and Hauber, 2005).

The ACC is also notable anatomically, as it sends a strong glutamatergic project to the dorsomedial striatum (DMS), an area that is crucial for goal-directed actions and the transition to addiction (McGeorge and Faull, 1989; Robbins & Everitt, 2002; Yin et al., 2005; Nonomura et al., 2018). To date, addiction research examining prefrontal cortical projections to striatum have not distinguished the circuits by cell-type specificity;

yet cortical projection neurons are comprised of two physically-intermingled but distinct groups of pyramidal neurons. The intratelencephalic (IT) neurons project bilaterally to striatum and to contralateral cortex whereas the pyramidal tract (PT) neurons project ipsilaterally to striatum, pyramidal tract, and several other downstream structures (Reiner et al., 2010; Shepherd 2013). In addition, these neuronal populations differ in several key ways, including morphology, receptor expression, and electrophysiological properties (Elliott et al., 2018; Shepherd 2013; Reiner et al., 2010). Given the breadth and magnitude of biological differences between these two cell types, we hypothesized that IT and PT neurons would have distinct contributions to behaviors related to cocaine addiction.

The inputs to IT and PT neurons have not been characterized, so we examined them using a modified rabies system for monosynaptic retrograde tracing (Wall et al, 2010). Cre-dependent viruses expressing an avian receptor to permit rabies infection (AAV5-EF1α-FLEX-TVA-mCherry) and a G-protein necessary for retrograde synaptic travel of rabies (AAV8-CA-FLEX-RG) were infused unilaterally into the ACC and a retrograde Cre-virus (CAV-Cre) was infused into the ipsilateral pyramidal tract or contralateral DMS to restrict AAV expression to PT or IT neurons, respectively. The modified rabies virus (EnvA G-deleted Rabies-eGFP) was then infused into the ACC, thus allowing for visualization of the direct projections to PT or IT neurons. We found that inputs to PT neurons were largely restricted to cortical neurons (Fig. 3.1A). In contrast, inputs to IT neurons were much more diverse, including an especially strong input from the thalamus (Fig. 3.1B).

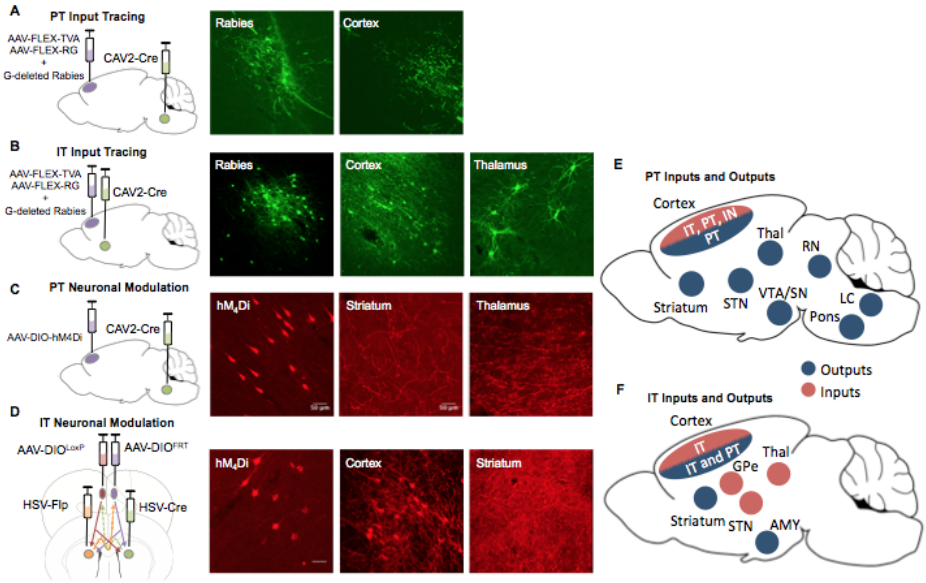

Figure 3.1. IT and PT neurons in ACC have distinct input and output partners. (A) To trace PT inputs, retrograde CAV-Cre virus was injected into the pyramidal tract and Cre-dependent TVA and RG vectors were infused into the ACC with the modified EnvA G-deleted rabies-eGFP. (B) To trace IT inputs, retrograde CAV-Cre virus was injected into the dorsomedial striatum and Cre-dependent TVA and RG vectors were infused into the ACC with the modified EnvA G-deleted rabies-eGFP. (C) To modulate PT neurons and examine PT outputs, retrograde CAV-Cre was injected into the pyramidal tract and a Cre-dependent hM4D$_i$ receptor was infused into the ACC. (D) To modulate IT neurons and examine IT outputs, retrograde HSV-Cre and HSV-Flp was injected into one side of dorsomedial striatum each and the complementary Cre-dependent hM4D$_i$ receptor and Flp-dependent hM4D$_i$ receptor were infused into the contralateral ACC. (E) PT neurons have diverse outputs, but a restricted source of cortical inputs. (F) IT neurons have a restricted set of outputs, but a diverse set of inputs.

To allow for transient modulation of cell-specific neural activity, we used a multi-recombinase-dependent Designer Receptor Exclusively Activated by Designer Drugs (DREADD) receptor approach. For selective expression in PT neurons, the inhibitory hM$_4$D$_i$ receptor (AAV8-hSyn-DIO-hM4D$_i$-mCherry) was bilaterally infused into the ACC and CAV-Cre was infused bilaterally into the pyramidal tract. For selective expression in IT neurons, the DIO-hM4D$_i$ virus was infused into one ACC hemisphere and HSV-Cre

was infused into the contralateral DMS. In addition, a Flp-dependent hM_4D_i virus (hSyn-FRT-$hM4D_i$) was infused into the other ACC hemisphere and the retrograde virus HSV-Flp was infused into the contralateral DMS (Fig. 3.1). The outputs of PT and IT neurons were visualized using the mCherry tag of the DIO-$hM4D_i$ virus. Consistent with other reports (Gerfen et al., 2013), PT terminal expression was observed in striatum, thalamus, etc. whereas IT terminal expression was only evident in the striatum and cortical layers (Fig. 3.1). Together with the input patterns, these data provide evidence for a cell-type specific split in corticothalamic loops with thalamic information being relayed to the cortex via IT neurons and cortical information being conveyed to the thalamus via PT neurons.

The reinforcing/rewarding effects of cocaine can be measured by a conditioned place preference (CPP) paradigm, where animals that receive pairings of cocaine on one side of a chamber will spend more time on that side when allowed to freely explore the chamber (Tzschentke 2007; Brabant et al., 2005). We examined the effect of transiently decreasing PT and IT cortical activity on the development of a CPP to cocaine to test the hypothesis that the neuronal populations would have opposing modulatory effects on cocaine reward. We found that animals spent significantly more time in the side of the chamber that had been paired with cocaine plus PT inhibition compared to the side of the chamber that had been paired with cocaine alone (p = 0.002; Fig. 3.2C). In addition, pairing cocaine with optogenetic activation of PT neurons led to a significant decrease in the amount of time spent in that side of the chamber (p = 0.019; Fig. 3.2D). Unexpectedly, IT inhibition had no effect on a CPP for cocaine (p = 0.069, Fig. 3.2E). The effects of PT neuronal modulation can be attributed to alterations

in drug reward because inhibition of PT neurons alone had no effect on chamber preference (p = 0.565, Figure 3.2F). This effect was specific to drug reward as inhibition of PT neurons did not affect the rewarding properties of a hedonic natural reward (p = 0.76, Fig. 3.2H).

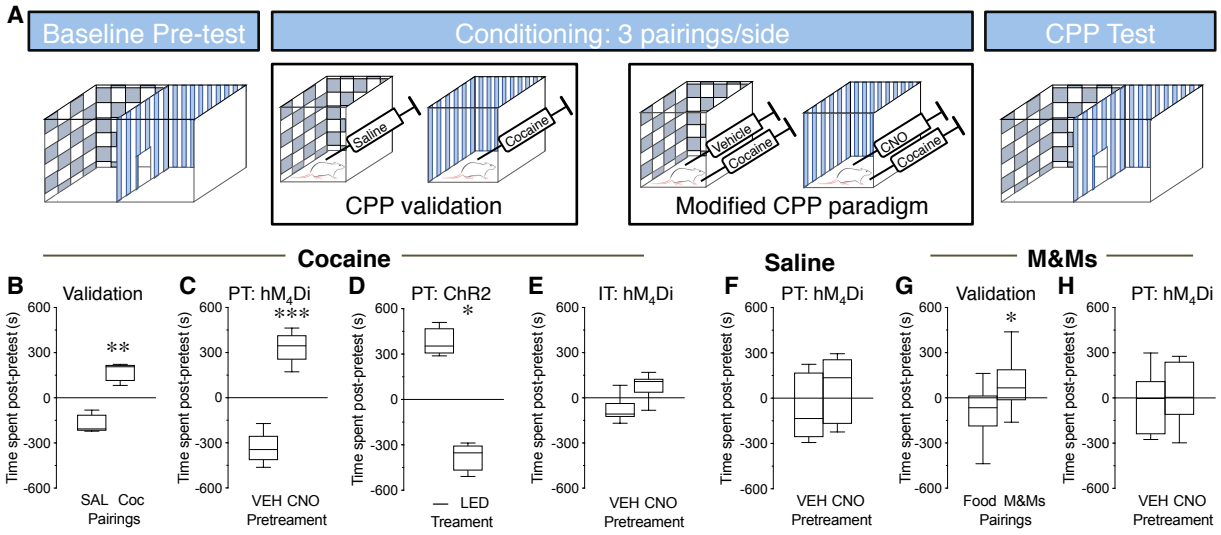

Figure 3.2. PT inhibition enhances the rewarding properties of cocaine. (A) During CPP validation, one chamber was paired with cocaine and the other chamber was paired with saline. For the modified CPP paradigm, cocaine was paired with both chambers and PT or IT inhibition was specific to one chamber in order to determine if they could modulate the rewarding properties of cocaine. (B) Cocaine is sufficient to produce a conditioned place preference (p = 0.012). (C) When both chambers were paired with cocaine, there was a significant preference for the chamber that had concurrent PT inhibition, indicating that PT inhibition enhances the rewarding properties of cocaine (p = 0.002). (D) There was a significant avoidance of a chamber associated with optogenetic stimulation of PT neurons when animals received cocaine in both chambers, indicating that stimulation of PT neurons is sufficient to reduce the rewarding properties of cocaine (p = 0.019). (E) When both chambers were paired with cocaine, there was no preference for the chamber that had concurrent IT inhibition, indicating that IT inhibition does not affect the rewarding properties of cocaine. (F) There was no preference for the PT inhibition chamber when animals received saline in both chambers, indicating that PT inhibition is not inherently rewarding. (G) A hedonic natural reward (M&M candies) was sufficient to produce a conditioned place preference. (H) There was no preference for the PT inhibition chamber when animals received M&M candies in both chambers, indicating that PT inhibition does not modulate the rewarding properties of a natural reinforcer.

The aversive properties of cocaine can be assessed using a conditioned taste avoidance (CTA) paradigm, where administration of high doses of cocaine following consumption of a sucrose solution will result in subsequent avoidance of the sucrose (Fig. 3.3A, E-G) (Hunt & Amit, 1987; Ferrari et al., 1991; Grigson 1997). We examined the effect of transiently decreasing PT and IT cortical activity on the development of a CTA to test the hypothesis that the neuronal populations would have opposing modulatory effects on the aversive effects of cocaine. We found that although PT neuron inhibition had no effect on cocaine-induced avoidance of sucrose consumption, inhibition of IT neurons significantly attenuated the reduction in sucrose consumption (p = 0.029; Fig. 3.3E-G). Interestingly, the amount of time spent drinking per bout was similar across groups; however, animals that received IT inhibition had significantly more drinking bouts (p = 0.004; Fig. 3.3F-G), indicating that inhibition of these neurons was acting to block the ability of cocaine to reduce approach behavior towards the sucrose solution. These data lend support to the notion that the CTA paradigm does indeed reflect a negative component of the drug experience, as a manipulation that greatly enhanced cocaine reward (PT inhibition) had no effect on a cocaine-induced CTA whereas a manipulation that did not alter the rewarding properties of cocaine (IT inhibition) did reduce the cocaine-induced CTA. This is in contrast to the idea that a cocaine CTA is driven by cocaine's strength as a reinforcer occluding the rewarding aspects of the sucrose (Grigson, 1997; Di Chiara et al., 2004).

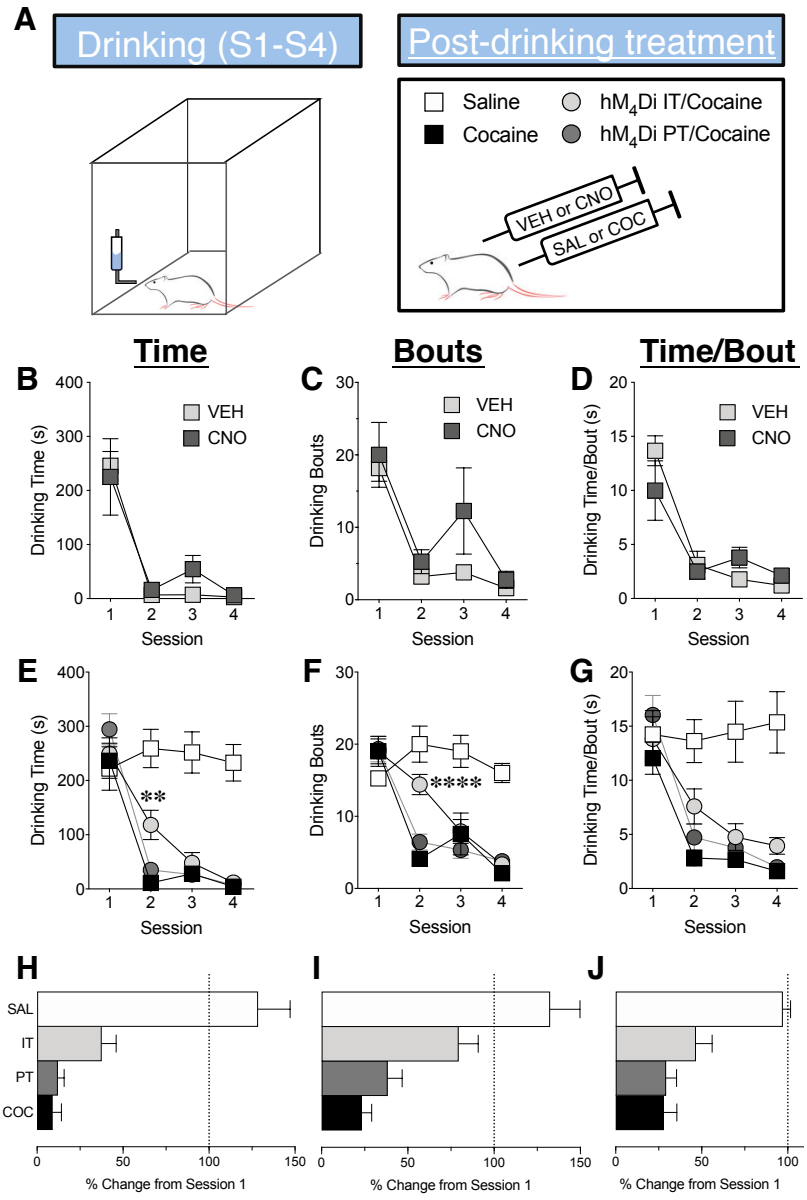

Figure 3.3. IT inhibition attenuates the aversive properties of cocaine. (A) After being exposed to a 15% sucrose solution for 30 minutes, animals received an injection of VEH or CNO followed 15 min later by cocaine or saline (B-D) In control animals (no hM_4D_i expression), CNO did not affect a cocaine-induced CTA (E) Cocaine pairing significantly reduces time spent drinking a sucrose solution ($p < 0.0001$). IT inhibition significantly attenuates the cocaine-mediated reduction in time spent drinking a sucrose solution ($p = 0.029$). (F) Cocaine pairing significantly reduces the number of drinking

bout of a sucrose solution ($p < 0.0001$). IT inhibition significantly attenuates the cocaine-mediated reduction in drinking bouts of a sucrose solution ($p = 0.003$). (G) Cocaine pairing significantly reduces the time spent drinking per bout of a sucrose solution ($p < 0.0001$). There was no effect of IT or PT inhibition on the time spent drinking per bout of a sucrose solution. (H-J) IT inhibition significantly attenuanted the cocaine-induced reduction in time spent drinking and drinking bouts on day two, but did not alter drinking time per bout.

In conclusion, we have identified distinct striatal-projecting cortical neurons (PT and IT) involved in regulating the positive and negative components of drug experience. This research provides the first evidence for the differential role of these cell populations in the reinforcing and aversive properties of cocaine and marks the first time that separate circuits (both anatomically and functionally) have been shown to exist among prefrontal cortex pyramidal neurons. Hypoactivity in the prefrontal cortex of drug addicts has long been considered a dangerous side effect of chronic drug use, due to a loss of executive control and an increase in impulsivity (Dackis & O'Brien, 2005; Volkow et al., 2004). Our data provides important new insights into the scope of this hypoactivity, as it suggests that a hypoactive prefrontal cortex would enhance the rewarding components of drug use (through a loss of PT activity) while simultaneously reducing the negative components (through a loss of IT activity). Consequently, these events would produce a significant shift in reward evaluation that favors continued drug use. This evidence of divergent roles for striatal-projecting cortical neurons in drug use, therefore, has fundamental implications for our understanding of cortical function in drug addiction.

C. Materials and Methods

Animal use.

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Use and Care Committee and adhered to National Institutes of Health (NIH) guidelines. Male Sprague Dawley rats (Envigo) weighing 250-274g upon arrival acclimatized to the environment for at least 3 days prior to any experimental manipulation. All rats were pair housed for the duration of the experiments. The housing environment was maintained on a 12h light/dark cycle and contained temperature and humidity control. Food and water was available *ad libitum*.

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 first dissolved in 100% DMSO, then diluted in sterile water for a final concentration of 5% DMSO. Vehicle injections were 5% DMSO in sterile water. Cocaine HCl (obtained from National Institute of Drug Abuse) was dissolved in sterile 0.9% saline and administered *ip* in a volume of 1 mL/kg at doses of 10 mg/kg (cocaine CPP) or 30 mg/kg (*sc*, cocaine CTA).

Viral vectors.

A Cre-dependent hM_4D_i-DREADD (AAV8-hSyn-DIO-hM_4D_i-mCherry) viral vector was developed by Bryan Roth (University of North Carolina) and packaged in adenoassociated virus (AAV, serotype 8) at Addgene with an approximate titer of 1 x 10^9 viral genomes per µL. A Flp-dependent hM_4D_i-DREADD (AAV1-hSyn-FRT-hM_4D_i-

YFP) was obtained from Larry Zweifel (University of Washington. A canine adenovirus expressing Cre-recombinase (CAV2-Cre) had a titer of approximately 2.5 x 10^9 viral genomes per mL and was prepared as previously described (Kremer et al., 2000) and obtained from John Neumaier (University of Washington). EnvA G-deleted rabies vector (EnvA G-deleted Rabies-eGFP) was developed by Ed Callaway (Salk Institute) and obtained from the Salk Institute Viral Vector Core with a titer of approximately 2.91 x 10^7. The Cre-dependent rabies glycoprotein (AAV8-CA-FLEX-RG) and the Cre-dependent TVA receptor (AAV5-EF1a-FLEX-TVAmCherry) were obtained from the University of North Carolina viral vector core with an approximate titer of 1 x 10^12. The Cre-dependent channelrhodopsin (AAV5-EF1a-DIO-hChR2(H134R)-EYFP) was obtained from the University of North Carolina. The HSV-Cre (hEF1α-EYFP-IRES-Cre) and HSV-Flp (hEF1α-EYFP-IRES-Flpo) viral vectors were obtained from Dr. Rachael Neve (Harvard) at an approximate titer of 3.5 x 10^9.

Surgical techniques.

During all surgical procedures, rats were anesthetized with isoflurane (4% induction, 2% maintenance, inhalation) and received meloxicam (1 mg/kg, *ip*) 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): anterior cingulate (ACC), +2.5, ±0.7, -1.9, 0.5; dorsomedial striatum (DMS), +0.2, ±2.0, -4.1, 1.0; pyramidal tract, -9.2, ±0.4, -9.8, 0.5. All viruses were bilaterally infused in the region of interest at a rate of 400 nL/min, and needles

were left in place for an additional 5 minutes to allow for diffusion away from the injection site. For targeting PT neurons, animals received CAV2-Cre infusions into the pyramidal tract (0.5 µL/side) and the complementary Cre-dependent virus in ACC (chemogenetic experiments: AAV8-hSyn-DIO-hM4D$_i$-mCherry, 0.5 µL/side; optogenetic experiments: AAV5-EF1a-DIO-hChR2(H134R)-EYFP, 0.5 µL/side; rabies tracing experiments: AAV8-CA-FLEX-RG (0.3 µL/side), AAV5-EF1a-FLEX-TVAmCherry (0.3 µL/side), and EnvA G-deleted Rabies-eGFP (0.5 µL/side)). For unilateral targeting of IT neurons, animals received CAV2-Cre infusions unilaterally into DMS (1.0 µL) and the complementary Cre-dependent virus in the contralateral side of ACC (chemogenetic experiments: AAV8-hSyn-DIO-hM4D$_i$-mCherry, 0.5 µL; rabies tracing experiments: AAV8-CA-FLEX-RG (0.3 µL), AAV5-EF1a-FLEX-TVAmCherry (0.3 µL), and EnvA G-deleted Rabies-eGFP (0.5 µL)). For bilateral targeting of IT neurons, animals received unilateral infusions of hEF1α-EYFP-IRES-Cre (1.0 µL) and hEF1α-EYFP-IRES-Flpo (1.0 µL) into DMS and the complementary Cre or Flp-dependent vector into the contralateral side of ACC (AAV1-hSyn-FRT-hM4D$_i$-YFP or AAV8-hSyn-DIO-hM4D$_i$-mCherry, 0.5 µL). Rats had at least 3 days of post-operative recovery and monitoring following stereotaxic infusions.

Rabies Tracing. Rats (n=4) received injections of helper viruses (AAV8-CA-FLEX-RG and AAV5-EF1a-FLEX-TVAmCherry) into ACC and CAV2-Cre either unilaterally into DMS (IT-targeting) or into pyramidal tract (PT-targeting). After 21d, the animals underwent stereotactic surgery to infuse EnvA G-deleted Rabies-eGFP into ACC. The rabies virus was allowed 7d to express before the rats were anesthetized with Beuthanasia-D (Patterson Veterinary) and transcardially perfused with PBS followed by

4% paraformaldehyde. Control animals underwent the same procedure, but did not receive any CAV2-Cre injections.

Conditioned Place Preference.

Rats (n = 85) underwent conditioned place preference (CPP) testing using a two-chamber apparatus (24 x 12 x 12 in, with a divider in the middle). The two chambers differed in their wall patterns (horizontal vs vertical stripes). Animals were first allowed to freely explore the apparatus for 20 min. Over the following three days, animals underwent six 20 min conditioning sessions (one in the morning and one in the afternoon separated by at least 4h). During these conditioning sessions, the rats were administered a treatment and restricted to one side of the chamber. The order that they received the treatments was counterbalanced each day. The day after the last set of conditioning sessions, animals were allowed to freely explore the chamber for 20 min. Time spent in each chamber during pre and post-conditioning test sessions were scored from videos by an experimenter blind to the conditions. A total of 14 rats were excluded from final analysis due either to a lack of viral expression (n = 13) or to not exploring both sides of the chamber during pre-test (n = 1).

For cocaine experiments, control animals received 10 mg/kg cocaine (*ip*) before being placed into one chamber and 0.9% saline (*ip*) before being placed into the other side of the chamber. For food experiments, control animals received M&Ms (4) in one side of the chamber with no food reward in the other chamber. Animals were previously exposed to M&Ms two days prior to behavioral testing to overcome neophobic suppression of eating. After validating that 10 mg/kg cocaine (*ip*) and M&M candies were sufficient to produce a conditioned place preference, we aimed to test the

contributions of IT and PT neurons to drug reward and natural rewards. In order to test drug reward, all rats received 10 mg/kg cocaine (*ip*) immediately prior to being placed into the chamber (both chambers) for these experiments. Both sides of the chamber would then be associated with cocaine and any manipulation of IT and PT neurons would act as a modulation of the conditioned reward induced by the cocaine (Fig. 2B). In order to test natural rewards, all rats received M&Ms in both sides of the chamber. Both sides of the chamber would then be associated with a food reward and any manipulation of IT and PT neurons would act as a modulation of the conditioned reward induced by the palatable food. For chemogenetic experiments, rats received CNO (5 mg/kg, *ip*) 30 min before being placed into one side of the conditioning chamber. For optogenetic experiments, animals received stimulation via head-mounted blue LED with parameters of 20 Hz, 20 ms pulse duration, for 60 sec with 30 sec of no stimulation in the ACC (for stimulation of PT cell bodies).

Conditioned Taste Aversion.

Control animals (n = 12) were placed into a novel cage and allowed 30 min to drink from a 15% sucrose solution. At the end of the session animals received vehicle injections (5% DMSO/95% sterile water, *ip*) or CNO injections (5 mg/kg, CNO, *ip*) and 15 min later either cocaine (30 mg/kg, *sc*) or saline (0.9%, *sc*). A separate group of animals (n = 8) received vehicle injections (5% DMSO/95% sterile water, *ip*) immediately after the the 30 min taste session with 15% sucrose follwed 15 min later by saline (0.9%, *ip*). In separate groups of animals (n = 22), we chemogenetically inhibited either IT or PT neurons (5 mg/kg, CNO, *ip*) immediately after the 30 min taste session with 15% sucrose followed 15 min later with cocaine (30 mg/kg, *sc*). Animals were

conditioned every other day for 4 sessions. 3 animals were removed from the experiment for failing to drink from the sucrose solution on the first day.

Virus Localization.

Rats were anesthetized with Beuthanasia-D and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight, and switched to PBS the next morning. Brains were sectioned in 40μM slices using a Leica vibrating microtome. Floating sections were washed in 0.2% Triton-X/PBS solution for 10 minutes and blocked in 0.2% Triton-X/PBS solution/5% normal goat serum for 30 minutes at room temperature. Sections were incubated in 0.2% Triton-X/PBS solution/5% normal goat serum/primary antibody against dsRed (1:400, rabbit host, Clontech, #632496) or GFP (1:400, mouse host, Millipore, MAB3580) while on a standard analog shaker (VWR) overnight at room temperature. Sections were rinsed three times in 0.2% Triton-X/PBS solution for 10 minutes. They were blocked in 0.2% Triton-X/PBS solution/5% normal goat serum for 30 minutes at room temperature. Then they were incubated in 0.2% Triton-X/PBS solution/5% normal goat serum/goat anti-rabbit Alexa568 conjugated secondary antibody (1:250, Invitrogen, A-11036) or goat anti-mouse Alexa488 conjugated secondary antibody (1:250, Invitrogen, A-11029) for 2 hours at room temperature on a standard analog shaker (VWR). Sections were rinsed in 0.2% Triton-X/PBS solution for 10 minutes followed by PBS for 10 minutes, mounted on slides, and cover slipped with Vectashield containing DAPI mounting medium (Vector Labs, H-1500). Z-stacks were captured using a Zeiss LSM 710 confocal microscope, and images were processed using ImageJ software (NIH).

Data Analysis.

All statistical analyses were determined prior to running experiments. GraphPad Prism 7 was used for statistical analyses. The effects of treatment and conditioning (pre- vs post-test) on time spent in each chamber was analyzed by taking a change score and running a one sample t-test for CPP experiments. The effects of time spent drinking, drinking bouts, and time spent drinking per bout were analyzed by repeated measures two-way analysis of variance (ANOVA) CTA experiments. For all comparisons, $\alpha \leq 0.05$.

Chapter 4

Pyramidal tract neurons in ACC transiently modulate cocaine sensitization, but do not influence drug-seeking

*This chapter is currently in preparation as an article for publication with Isah G. Webb, Michelle B. Seo, Dana G. Haberkorn, and Susan M. Ferguson as co-authors.

AFG, IGW, MBS, and DGH performed the behavioral experiments. AFG performed c-Fos experiment and data analysis. AFG and SMF designed the experiments and wrote the manuscript.

A. Abstract

Prefrontal cortex is a critical node of the motivational and reward circuitry that is altered during drug use. Corticolimbic projections play an important role in modulating drug-seeking under many different circumstances. However, little has been done to parse these projections out in a cell-type specific manner and determine their contributions to addiction behaviors. Here, we specifically examine pyramidal tract (PT) type neurons of the anterior cingulate, which project to the striatum as well as several other regions in the corticolimbic circuitry. Chemogenetic inhibition of PT-type neurons transiently enhances cocaine-induced sensitization, but does not affect the motivation to obtain cocaine or drug-seeking on a reinstatement test.

B. Introduction

Drug addiction is a chronic, debilitating disease marked by an enhanced motivation to take drugs, craving for drugs, and a prolonged susceptibility to relapse (Robinson and Berridge, 2000; Kalivas and O'Brien, 2008; Everitt et al., 2008). Cortical inputs to striatum play an important role in driving drug-seeking (Capriles et al., 2003; McFarland and Kalivas, 2001). Infralimbic cortex and prelimbic cortical projections to striatum are both important for regulating drug-seeking (Stefanik et al., 2013; Martin-Garcia et al., 2014; Stefanik et al., 2016; Augur et al., 2016; Ma et al., 2014). The infralimbic and prelimbic cortices both primarily project to the nucleus accumbens, though (McGeorge and Faull, 1989). Over time the importance of the striatal subregions shifts from the ventral striatum to dorsal striatum (Everitt et al., 2008). The ACC sends a dense projection to the DMS; however, little is known about the role of the ACC in drug-seeking or the motivation to obtain drug. The ACC is robustly activated by cocaine and cocaine associated cues (Zahm et al., 2010; Neisewander et al., 2000; Zhou et al., 2014).

Projections from cortex to striatum also play a necessary role in locomotor sensitization induced by drugs and are believed to be an important node of the circuitry underlying drug-craving (Kerstetter et al., 2016; Vanderschuren and Kalivas, 2000; Pierce et al., 1997). Most studies investigating the contributions of corticostriatal projections to addiction behaviors do so in a region specific manner. There are no studies parsing out corticostriatal circuits in a cortical cell-type specific manner. There are two types of cortical neurons that project to the striatum: the intratelencephalic (IT-type) neurons and pyramidal tract (PT-type) neurons. The projections of the IT-type

neurons are restricted to the cortex and the striatum. In contrast, the PT-type neurons project widely to several nodes of the corticolimbic circuitry, including the striatum, thalamus, ventral tegmental area, and subthalamic nucleus (Gerfen et al., 2013). The ability of PT-type neurons to communicate with multiple regions of the corticolimbic circuitry simultaneously make them good candidates for being responsible for many of the changes in cortical circuitry produced by drug use.

In order to test the role of PT-type neurons in addiction behaviors we used a dual viral chemogenetic technique to selectively target and modulate PT neurons in the ACC during locomotor sensitization as well as the motivation to obtain drug and drug-seeking induced by cues and a cocaine-prime. PT inhibition differentially affects the early and late stages of cocaine-induced locomotor sensitization. It suppresses locomotor activity to early cocaine administration, but ultimately enhances locomotor sensitization in a transient manner that is no longer extant on a future cocaine challenge session. Inhibition of PT-type neurons does not affect the motivation to obtain cocaine on a progressive ratio test or susceptibility to relapse on cue or cocaine-induced reinstatement tests.

C. Materials and Methods

Animal use.

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Use and Care Committee and adhered to National Institutes of Health (NIH) guidelines. Male Sprague Dawley rats (Envigo) weighing 250-274g upon arrival acclimatized to the environment for at least 3 days prior to any experimental manipulation. All rats were pair housed for the duration of the experiments. The housing environment was maintained on a 12h light/dark cycle and contained temperature and humidity control. Food and water was available *ad libitum*.

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 first dissolved in 100% DMSO, then diluted in sterile water for a final concentration of 5% DMSO. Vehicle injections were 5% DMSO in sterile water. Cocaine HCl (obtained from National Institute of Drug Abuse) was dissolved in sterile 0.9% saline and administered *ip* in a volume of 1 mL/kg at a dose of 15 mg/kg (cocaine sensitization), 10 mg/kg (cocaine-prime reinstatement), or *iv* at a concentration of 0.4 mg/kg/inf (cocaine self-administration).

Viral vectors.

A Cre-dependent hM_4D_i-DREADD (AAV8-hSyn-DIO-hM_4D_i-mCherry) viral vector was developed by Bryan Roth (University of North Carolina) and packaged in adenoassociated virus (AAV, serotype 8) at Addgene with an approximate titer of 1 x

10^9 viral genomes per µL. A canine adenovirus expressing Cre-recombinase (CAV2-Cre) had a titer of approximately 2.5 x 10^9 viral genomes per mL and was prepared as previously described (Kremer et al., 2000) and obtained from John Neumaier (University of Washington).

Surgical techniques.

During all surgical procedures, rats were anesthetized with isoflurane (4% induction, 2% maintenance, inhalation) and received meloxicam (1 mg/kg, *ip*) 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): anterior cingulate (ACC), +2.5, ±0.7, -1.9, 0.5; dorsomedial striatum (DMS), +0.2, ±2.0, -4.1, 1.0; pyramidal tract, -9.2, ±0.4, -9.8, 0.5. All viruses were bilaterally infused in the region of interest at a rate of 400 nL/min, and needles were left in place for an additional 5 minutes to allow for diffusion away from the injection site. For targeting PT neurons, animals received CAV2-Cre infusions into the pyramidal tract (0.5 µL/side) and the complementary Cre-dependent virus in ACC (AAV8-hSyn-DIO-hM4D$_i$-mCherry, 0.5 µL/side). Rats had at least 3 days of post-operative recovery and monitoring following stereotaxic infusions.

Locomotor Sensitization.

Fourteen days following stereotaxic surgery, rats (n = 47) received 7 injections of either cocaine (15 mg/kg, *ip*) or saline over 14d (one injection every other day). Thirty minutes prior to each injection, rats received either CNO (5 mg/kg, *ip*) or vehicle (5%

DMSO in sterile water). Rats were placed into locomotor activity boxes (San Diego Instruments) and ambulations were recorded as two consecutive infrared beam breaks over 60 min. After a 14d withdrawal period, animals underwent a challenge session where they received a 30 min habituation to the locomotion chambers followed by an *ip* injection of saline to test for a conditioned response. Thirty minutes later, rats received cocaine (10 mg/kg, *ip*) in the absence of CNO; behavior was recorded for 60 min to test for the persistence of sensitization. Nine rats were excluded from analysis because of a lack of viral expression.

Self-administration chambers.

Self-administration occurred in standard operant chambers (Med Associates) equipped with two retractable levers, two white stimulus lights, one located above each lever, white house light on the back of the chamber, house fan, and metal grid floor. A syringe pump outside the box delivered cocaine via tubing attached to a suspended swivel and the catheter backport allowing for free movement of the animal.

Self-administration procedure.

At least 5 days after surgery, rats were trained to lever press for cocaine on an FR1 schedule for 5 days. The training session began with the insertion of the two retractable levers. A lever press on the active lever resulted in a cocaine infusion (0.4 mg/kg/inf in 50 µL over 2.8s) and illumination of the white stimulus light above the active lever (4s). Additional presses on the active lever during the cue light presentation were recorded, but did not result in additional infusions. Pressing on the inactive lever had no consequences. The location of the active lever was counterbalanced across animals. The session ended after 3h or after the animal had received 10 infusions, whichever

came first. Following completion of 5 days of FR1 training, animals began 14 sessions (one session per day for 14 days) of intermittent (n = 41) access to cocaine. A single intermittent access session consisted of a 5 minute access to cocaine period followed by a 25 minute drug unavailable period. Levers were retracted during the drug unavailable period. This cycle repeated for a total of 155 minutes (6 drug available periods separated by 5 drug unavailable periods).

Progressive ratio.

At the conclusion of the self-administration sessions, animals underwent a progressive ratio test to determine their motivation to obtain drug. During the progressive ratio test, the number of responses required to earn each successive cocaine infused increased using the following progression: 1, 5, 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585. The session was terminated after 5h or when 1h elapsed since the last completed ratio. Half of the animals received CNO (5 mg/kg, *ip*) and half received vehicle (5% DMSO / 95% sterile water) 30 minutes before the start of the session. Animals then underwent 3 additional sessions of intermittent access to cocaine in order to re-establish their baseline before undergoing a second progressive ratio test before which they received the treatment (CNO or vehicle) that they had not received during the first session.

Extinction.

Rats underwent extinction at the conclusion of the progressive ratio sessions. Rats were put into the operant chambers for 60 minutes and responses on the active

and inactive lever had no consequences (i.e. no infusion of drug or illumination of cue light). Rats underwent extinction training for a total of 10 extinction sessions.

Cue-induced reinstatement.

At the conclusion of extinction, animals underwent a 60 minute cue-induced reinstatement test. Animals were put into the operant chamber and the session began with the extension of the levers and the illumination of the cue light above the active lever. Pressing on the active lever resulted in a 4 sec presentation of the cue-light, but no infusion of drug. Half of the animals received CNO (5 mg/kg, *ip*) and half received vehicle (5% DMSO / 95% sterile water) 30 minutes before the start of the session. After the cue-induced reinstatement test, animals underwent 5 additional sessions of extinction.

Cocaine-induced reinstatement.

After animals had re-extinguished their active pressing, a subset of animals (n = 31) underwent a cocaine-prime reinstatement test. Animals received an priming injection of cocaine (10 mg/kg, *ip*) and were put into the operant chamber. Levers were presented for this session, but responding did not have any programmed consequences (i.e. no infusions or light cue was given). Half of the animals received CNO (5 mg/kg, *ip*) and half received vehicle (5% DMSO / 95% sterile water) 30 minutes before the start of the session.

Cocaine-induced c-Fos validation of hM4D$_i$ receptor.

A subset of animals (n = 19) were perfused 15 min after the end of the cocaine-induced reinstatement session (75 min after cocaine injection and the start of the

session) with 4% PFA. Brains were removed, processed for immunohistochemistry, and c-Fos+ cells in the ACC were counted by an experimenter blind to the conditions.

Virus localization.

Rats were anesthetized with Beuthanasia-D and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight, and switched to PBS the next morning. Brains were sectioned in 40μM slices using a Leica vibrating microtome. Floating sections were washed in 0.2% Triton-X/PBS solution for 10 minutes and blocked in 0.2% Triton-X/PBS solution/5% normal goat serum for 30 minutes at room temperature. Sections were incubated in 0.2% Triton-X/PBS solution/5% normal goat serum/primary antibody against dsRed (1:400, rabbit host, Takara Bio Clontech, #632496) or mCherry (c-Fos experiment; 1:400, mouse host, Takara Bio Clontech, #632543) and c-Fos (1:400, rabbit host,Santa Cruz Biotechnology, sc-52) and while on a standard analog shaker (VWR) overnight at room temperature. Sections were rinsed three times in 0.2% Triton-X/PBS solution for 10 minutes. They were blocked in 0.2% Triton-X/PBS solution/5% normal goat serum for 30 minutes at room temperature. Then they were incubated in 0.2% Triton-X/PBS solution/5% normal goat serum/goat anti-rabbit Alexa568 conjugated secondary antibody (1:250, Invitrogen, A-11036) or goat anti-mouse Alexa568 conjugated secondary antibody (c-Fos experiment; 1:250, Invitrogen, A-11004) and goat anti-rabbit Alexa488 conjugated secondary antibody (c-Fos experiment; 1:250, Invitrogen, A-11034) for 2 hours at room temperature on a standard analog shaker (VWR). Sections were rinsed in 0.2% Triton-X/PBS solution for 10 minutes followed by PBS for 10 minutes, mounted on slides, and cover slipped with Vectashield containing DAPI

mounting medium (Vector Labs, H-1500). Z-stacks were captured using a Zeiss LSM 710 confocal microscope, and images were processed using ImageJ software (NIH).

Data analysis.

All statistical analyses were determined prior to running experiments. GraphPad Prism 7 was used for statistical analyses. Paired t-tests were used to analyze the effect of CNO vs vehicle on the progressive ratio break and active lever presses. An unpaired t-test was used to compare the effect of CNO vs vehicle on the active lever presses during the cue-induced and cocaine-primed reinstatement tests. A one-way repeated measures ANOVA was used to ensure that extinction resulted in a significant suppression of active presses. For locomotor sensitization experiments, ambulations across sessions as well as individual sessions were analyzed using repeated measures two-way ANOVA for time X CNO treatment (CNO vs vehicle) as well as time X cocaine treatment (cocaine vs saline). For the challenge session, ambulations were analyzed using a two-way ANOVA of CNO history (CNO vs vehicle during sensitization sessions) X cocaine history (cocaine vs saline during sensitization sessions). Differences in the number of c-Fos+ cells in the ACC were tested using an unpaired t-test. For all comparisons, $\alpha \leq 0.05$.

D. Results

PT inhibition transiently enhances locomotor sensitization

Locomotor sensitization is a valuable model for examining the long-term sensitizing effects of drugs of abuse (Robinson and Berridge, 1993; White and Kalivas, 1998). We tested the ability of chemogenetic inhibition of PT-type neurons in the ACC to affect locomotor sensitization. We found that animals that received PT inhibition had a significantly enhanced cocaine-induced sensitization (two-way RM ANOVA, main effect of time $F_{(6,126)} = 12.12$, $p < 0.0001$; no effect of PT inhibition $F_{(1,21)} = 0.594$, $p = 0.45$; main effect of cocaine x PT inhibition interaction $F_{(6,126)} = 2.277$, $p = 0.04$, Fig. 4.1C). Upon further examination, we discovered that PT inhibition was having its greatest effect early in the sessions, and actually caused a reduced locomotor effect during the second session of cocaine administration and a significantly greater effect by the last day of sensitization (session 2: two-way RM ANOVA, main effect of time $F_{(59,1239)} = 26.42$, $p < 0.0001$; no effect of PT inhibition $F_{(1,21)} = 0.647$, $p = 0.43$; main effect of time x PT inhibition interaction $F_{(59,1239)} = 2.35$, $p < 0.0001$; session 7: two-way RM ANOVA, main effect of time $F_{(59,1239)} = 19.53$, $p < 0.0001$; no effect of PT inhibition $F_{(1,21)} = 2.335$, $p = 0.141$; main effect of time x PT inhibition interaction $F_{(59,1239)} = 1.899$, $p < 0.0001$, Fig. 4.2A and 4.2C). This indicates that inhibition of PT neurons in the ACC has opposing effects on early cocaine experience (attenuation) and long-term cocaine administration (enhancement). There were no group differences during habituation or pre-treatment (habituation: two-way RM ANOVA, main effect of time $F_{(6,114)} = 33.18$, $p < 0.0001$; no effect of PT inhibition $F_{(1,19)} = 0.634$, $p = 0.436$; no effect of cocaine x PT inhibition interaction $F_{(6,114)} = 0.608$, $p = 0.723$; pretreatment: two-way RM ANOVA, no

effect of time $F_{(6,114)}$ = 1.567, p = 0.163; no effect of PT inhibition $F_{(1,19)}$ = 1.847, p = 0.19; no effect of cocaine x PT inhibition interaction $F_{(6,114)}$ = 0.883, p = 0.51, Fig. 4.1A and 4.1B). The reduced locomotion over time during the habituation phase is likely due to a loss of novelty indicating that the animals had habituated to the locomotion chambers.

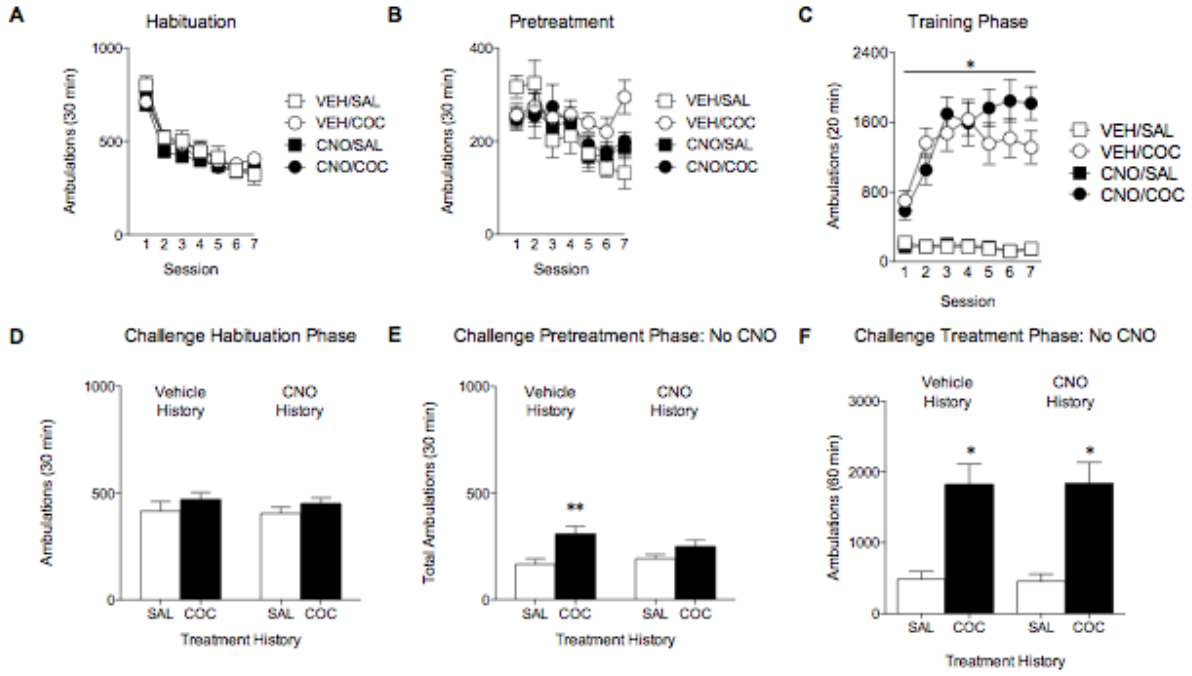

Figure 4.1. PT inhibition produces a transient enhancement of cocaine-induced locomotor sensitization. (A) There were no group differences in locomotion during habituation. (B). There were no group differences in locomotion during pre-treatment. (C) PT inhibition significantly enhanced cocaine-induced locomotor sensitization (p = 0.04). (D) There was no difference in habituation during the challenge session. (E) Animals with a history of VEH/COC developed a significant anticipatory conditioned response during the pre-treatment phase, which was absent in animals that had a history of PT inhibition (p = 0.003). (F) Animals that had received cocaine previously had significantly higher locomotion on challenge (p < 0.0001), but there was no effect of PT inhibition.

A challenge session 14 days after the final sensitization session revealed that both groups of animals receiving cocaine had sensitized; however, there were no group differences between animals receiving CNO and vehicle (two-way ANOVA, main effect

of cocaine $F_{(1,34)}$ = 32.19, p < 0.0001; no effect of PT inhibition history $F_{(1,34)}$ = 0.002, p = 0.969; no effect of cocaine x PT inhibition history interaction $F_{(1,34)}$ = 0.007, p = 0.935, Fig. 4.1F and 4.2D). There was no difference between groups during habituation (two-way ANOVA, no effect of cocaine $F_{(1,34)}$ = 2.213, p = 0.146; no effect of PT inhibition history $F_{(1,34)}$ = 0.202, p = 0.656; no effect of cocaine x PT inhibition history interaction $F_{(1,34)}$ = 0.016, p = 0.901, Fig. 4.1D). There was a significant increase in locomotion during the pretreatment phase caused by previous cocaine treatment (two-way ANOVA, main effect of cocaine $F_{(1,34)}$ = 10.20, p = 0.003; no effect of PT inhibition history $F_{(1,34)}$ = 0.285, p = 0.597; no effect of cocaine x PT inhibition history interaction $F_{(1,34)}$ = 1.883, p = 0.179, Fig. 4.1E). Post-hoc tests revealed that while animals with a history of vehicle treatments had a significant increase during this phase, animals that had a history of PT inhibition did not. This indicates that vehicle animals had obtained an anticipatory conditioned response, which was absent in the PT inhibition animals.

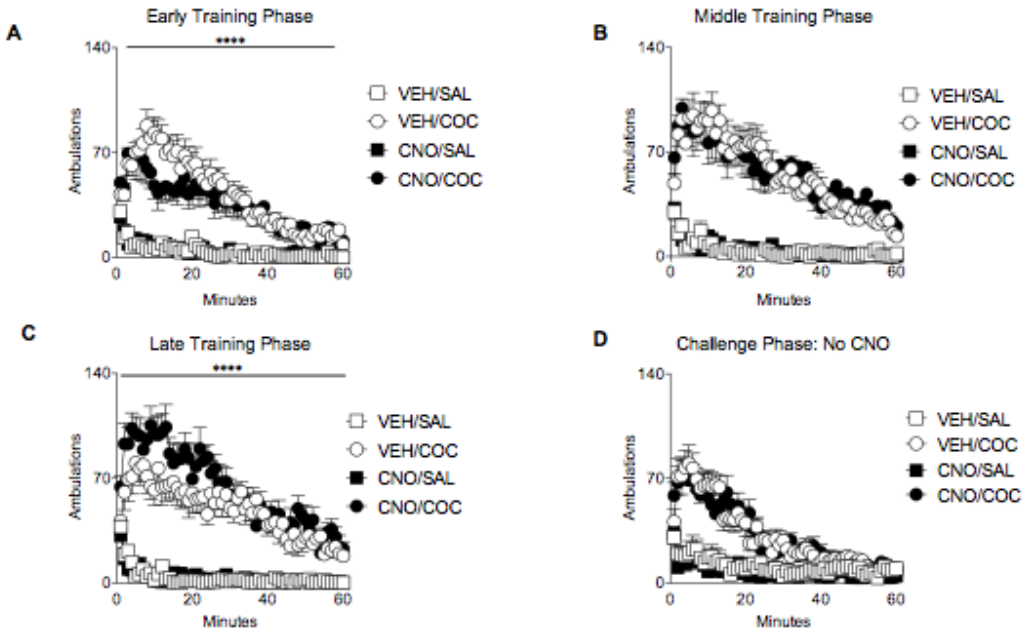

Figure 4.2. PT inhibition has opposing effects on cocaine sensitization in early versus late phase training. (A) During an early sensitization session, PT inhibition reduces the amount of cocaine-induced locomotor activity early in the session (p < 0.0001). (B) During the middle of sensitization training, PT inhibition has no discernible effect on cocaine-induced locomotion. (C) At the end of sensitization training, PT inhibition enhances cocaine-induced locomotion early in the session (p < 0.0001). (D) During the challenge session there was no difference between animals that had previously received PT inhibition versus those that had not.

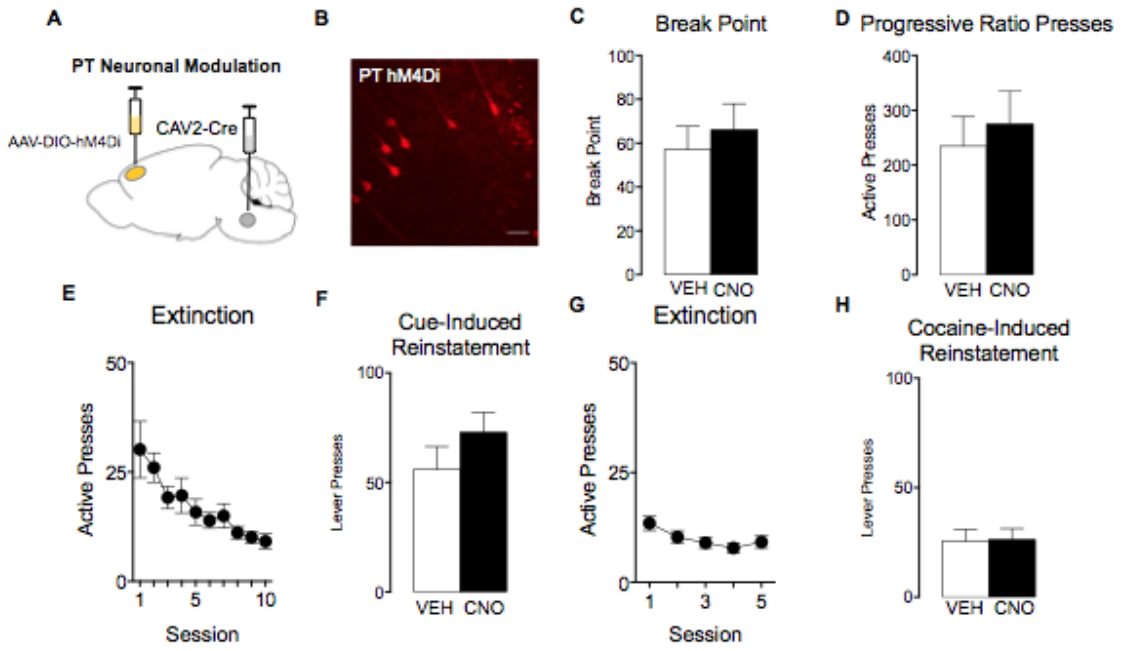

Figure 4.3. PT inhibition does not affect motivation to obtain cocaine or reinstatement. (A) A retrograde CAV-Cre viral vector was infused into the pyramidal tract and a Cre-dependent $hM4D_i$ virus was injected into the ACC. (B) Expression of the $hM4D_i$ receptor in PT-type neurons in the ACC. Scale bar = 50 μM (C) PT inhibition failed to modulate break point for cocaine. (D) PT inhibition failed to alter active presses during the progressive ratio test. (E) Animals extinguished lever pressing following progressive ratio tests. (F) PT inhibition did not alter a cue-induced reinstatement test. (G) Animals re-extinguished lever pressing following cue-induced reinstatement. (H) PT inhibition did not alter lever pressing during a cocaine-prime reinstatement test.

PT inhibition does not alter progressive ratio

Following self-administration, we tested the ability of PT inhibition to alter the motivation to obtain cocaine by using a progressive ratio test. We found that there was no difference in lever presses between the first and second progressive ratio test, indicating that a within subject design was reasonable for this experiment (*t* test, t_{30} = 1.496, p = 0.145, data not shown). Inhibition of PT neurons in the ACC did not have any

effect on the motivation to obtain drug as measured by the break point achieved or the number of active lever presses (break point: *t* test, t_{30} = 0.734, p = 0.469; lever presses: *t* test, t_{60} = 0.59, p = 0.559, Fig. 4.3C and 4.3D).

PT inhibition does not affect cue-induced reinstatement

In order to determine the ability of PT inhibition to affect the ability of a salient drug-associated cue to induce relapse, we performed a cue-induced reinstatement test. We found that PT inhibition had no effect on the active lever presses during the cue-induced reinstatement session (*t* test, t_{29} = 1.211, p = 0.236; Fig. 4.3F).

PT inhibition does not affect cocaine-primed reinstatement

After re-extinguishing their active lever pressing, we examined the role of PT neurons during a cocaine-primed reinstatement. We found that PT inhibition had no effect on the active lever presses during the reinstatement session (*t* test, t_{23} = 0.076, p = 0.934; Fig. 4.3H).

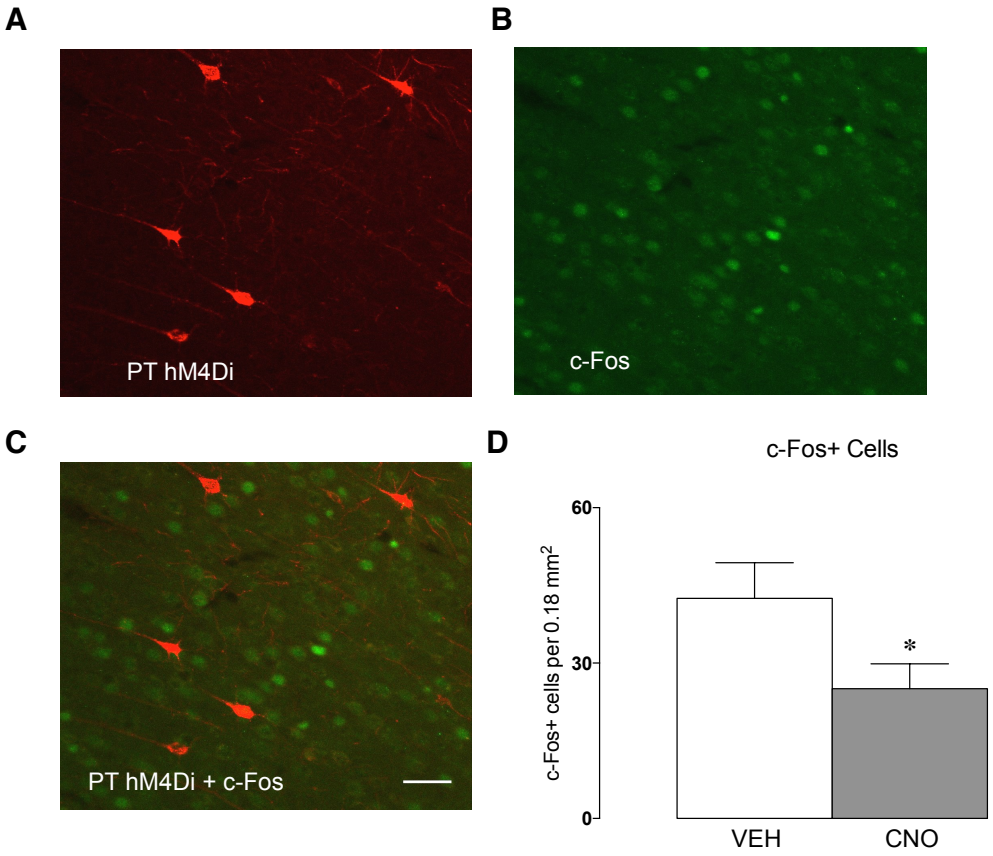

Figure 4.4. DREADD mediated reduction of cocaine-induced c-Fos+ cells in ACC. **(**A) $hM4D_i$ expression in PT-type neurons in ACC. (B) c-Fos+ neurons in ACC following cocaine-induced reinstatement. (C) Merge of images demonstrating the lack of c-Fos in neurons that were chemogenetically inhibited. (D) Chemogenetic inhibition significantly reduced the number of c-Fos+ cells in ACC following cocaine-induced reinstatement (*p* = 0.049). Scale bar = 50 μM

Chemogenetic inhibition of PT neurons reduces cocaine-induced c-Fos in the ACC

CNO activates $hM4D_i$ receptors, which decreases neuronal activity, likely through decreases in cAMP and the opening of GIRK channels (Armbruster et al., 2007; Ferguson et al., 2011). This manipulation has also been shown to be effective in the PFC (Kerstetter et al., 2016; Katzel et al., 2014; Robinson et al., 2014; Kozorovitskiy et al., 2012). As this is the first report examining PT-type neurons in drug addiction and the

use of chemogenetic techniques in PT neurons, we sought to validate our technique through the use of a c-Fos experiment. c-Fos is an immediate early gene that is expressed after neuronal activation (Sheng and Greenberg, 1990). We found that CNO-induced activation of the $hM4D_i$ receptor in PT-type neurons reduced the number of cocaine-induced c-Fos+ cells in ACC by ~40%, which was significant (t_{17} = 2.114, p = 0.049, Fig. 4.4D).

E. Discussion

Cortical projections to striatum play an important role in addictive behaviors following drug use; however, there have been no attempts thus far to describe the contributions of different cortical cell types that project to striatum. Here, we characterize, for the first time, the role of PT-type neurons in the ACC on the motivation to obtain drug, cue and cocaine-induced drug-seeking, and cocaine-induced psychomotor sensitization. PT neurons are large pyramidal neurons with dense arborization patterns that have a promiscuous projection pattern characterized by outputs to several structures in the corticolimbic circuitry (Morishima and Kawaguchi, 2006, Dembrow et al., 2010, Suter et al., 2013, Mason and Larkman, 1990, Hattox and Nelson, 2007, Miller et al., 2008, Gee et al., 2012, Shepherd, 2013; Gerfen et al., 2013; Reiner et al., 2010). The corticolimibic circuitry is notable for underlying a wide array of motivated and affective behaviors, including addictive behaviors (Nestler et al., 2001; Robinson and Berridge, 1993). Given the importance of their projection partners, we hypothesized that these neurons were likely to play an important role in drug addiction. Inhibition of PT neurons in ACC differentially affected early non-contingent cocaine administration, extended non-contingent cocaine administration, and cocaine-seeking.

When paired with non-contingent cocaine injections, PT inhibition, which should result in reduced cortical glutamatergic excitation, produced an enhancement of locomotor sensitization. This is an interesting departure from previous findings on the role of prefrontal glutamate in cocaine sensitization. Prefrontal glutamate release seems to play an important role in cocaine-induced sensitization more than to other drugs (Vanderschuren and Kalivas, 2000). Cocaine sensitization results in enhanced

locomotion and enhanced glutamate release (Reid and Berger, 2006). Systemic glutamate antagonists as well as lesions of the prefrontal cortex prevent locomotor sensitization induced by cocaine (Li et al., 1999). These previous findings largely represent less specific manipulations either affecting glutamate generally or all of the prefrontal cortex. Combined with our data this indicates that the PFC does not have a single role in cocaine-induced locomotor sensitization. It could be that there are competing signaling pathways within the prefrontal cortex and when the entire PFC is lesioned, the net result is one that overwhelms the loss of just the PT neurons, which we expect would enhance sensitization. It is worth noting that the enhancement of PT inhibition on locomotor sensitization was a transient one that was not apparent two weeks later on a challenge session. This finding implies that the long-term synaptic and molecular alterations that may be occuring in the corticolimbic circuitry are likely not occurring in the PT neurons or that our manipulation had no effect on long-term plasticity.

Additionally, we found a contradictory effect when examining the early sessions of cocaine administration. In an early cocaine session, PT inhibition played a restrictive role, attenuating the locomotor movement. There is significant evidence that the anatomical substrates underlying short-term and long-term cocaine sensitization are different (Vanderschuren and Kalivas, 2000). For instance, during short-term sensitization PFC neurons have reduced sensitivity to dopamine and ventral striatal neurons have reduced sensitivity to glutamate, but these effects are transient (Vanderschuren and Kalivas, 2000). It is possible that modulation of PT neuron activity has differing effects over time because of the interaction of glutamate downstream in

the striatum with other neurotransmitter systems that are known to change during sensitization, such as dopamine release or GABA signaling (Vanderschuren and Kalivas, 2000; Kalivas and Duffy, 1993; Resnick et al., 1999). The lack of effect on challenge does indicate that the chemogenetic manipulation was insufficient to alter the long-term plasticity that is typically associated with locomotor sensitization.

The inability of PT-type neurons to modulate the motivation to obtain drug and the susceptibility to relapse was a surprising finding given the importance of corticostriatal projections in these aspects of drug addiction. It is possible that after drug use transitions to compulsive seeking and taking, it may be driven more intensely by the negative aspects associated with addiction (Koob and Le Moal, 2001). If this is true, it is possible that IT neurons may play an important role than PT neurons in these behaviors. One well-known side effect of chronic drug use is a basal hypoactivity of prefrontal cortex. In this set of experiments, PT inhibition did not occur until after the animals had already undergone an intermittent access self-administration paradigm. The intermittent access self-administration paradigm has been found to more strongly induce addictive behaviors than continuous access models (Zimmer et al., 2012). It is possible that PT neuron activity was already significantly depressed following the completion of this paradigm and the manipulation was not capable of driving activity low enough to have a functional effect. The self-administration experiments are also unique compared to the sensitization experiment in that the animals have significant drug-taking experience before they ever receive the PT manipulation. In this design, the PT modulation is restricted to the drug-seeking tests, but the PT neurons of ACC may be more involved with the drug-taking experience. It is possible that an attenuation of PT

neurons may have more effect on a consumptive task as opposed to a seeking based task. For instance, it is possible that reduced activity of PT-type neurons may play a role in behaviors where drug is actively being taken and consumed, such as in binge-type behaviors. If this were true, it is still conceivable that PT neurons could indirectly affect drug-seeking. If PT neurons play a role in the drug-taking experience, they may modulate the reinforcing components of the consumptive portion of self-administration, thereby later influencing future seeking. Modulation of PT neurons in ACC could be capable of modulating susceptibility to addiction, but incapable of affecting behaviors after the habitual and compulsive behaviors associated with addiction have set in.

Cocaine and cocaine cues induce robust expression of the immediate early gene, c-Fos, in the ACC (Zahm et al., 2010; Neisewander et al., 2000; Zhou et al., 2014). Furthermore, the $hM4D_i$ receptor is capable of reducing neuronal activation in the PFC (Katzel et al., 2014; Kerstetter et al., 2016; Kozorovitskiy et al., 2012; Robinson et al., 2014). However, as this is the first investigation of PT-type neurons in the ACC in self-administration, we verified that the chemogenetic technique used was suitable to reduce neuronal activation. The significant reduction is cocaine-induced c-Fos in the ACC indicates that CNO activation of the $hM4D_i$ receptor was sufficient to reduce neuronal activity in the ACC. It seems likely that this is occurring through a mechanism involving a reduction in cAMP and opening of GIRK channels (Armbruster et al., 2007; Ferguson et al., 2011).

In conclusion, these results indicate that ACC PT neurons play a complicated role in addiction behaviors that is not purely facilitatory or restrictive. When paired with an acute cocaine injection, the effect is an attenuation of motor activity, but over time

this pairing gives way to a transient enhancement of locomotor sensitization. Following intermittent access self-administration, inhibition of PT neurons did not affect the motivation to obtain drug or susceptibility to reinstate. This may indicate that PT-type neurons in the ACC play an important role in the drug-taking experience and the effects associated with it when cocaine is on board, but does not affect the desire to seek drug.

Chapter 5

Conclusions and Future Directions

In this thesis, I explored two main issues: the role of the temporal pattern of drug intake in addiction and the contributions of different striatal-projecting cortical neuronal subtypes in addiction. First, I demonstrated that an intermittent access self-administration paradigm produced a meaningful variability in addiction behaviors that was consistent with individual differences in addiction susceptibility. I then demonstrated IT and PT neurons in the ACC differentially participate in the positive and negative aspects of cocaine use. Finally, I showed that in addition to its role in the reinforcing aspects of cocaine, PT inhibition transiently enhances locomotor sensitization, but does not alter drug-seeking or the motivation to obtain drug.

In chapter 2, I sought to characterize the differences in addiction behaviors produced by an intermittent access self-administration model. Although this model has previously been found to increase the motivation for drug, there are many other components of addiction that have never been studied with relation to intermittent access self-administration (Zimmer et al., 2012). I found that intermittent access self-administration produced a subset of animals that were enhanced on several addiction behaviors. Individual differences are an important and often overlooked aspect of drug addiction, as only a relatively small population of people who try drugs ever become addicts (Anthony et al., 1994; Penberthy et al., 2010; Grant and Dawson, 1998). Intermittent access self-administration produces a subset of animals that escalate their

intake over time, have higher susceptibility to cue-induced reinstatement, engage in binge-like patterns of consumption and seeking, and have increased cocaine-induced sensitization. In total, intermittent access produced a more reliable and coherent model of addiction than continuous access in which high scores on any one facet of addiction was not predictive of any other facet.

Going forward, it would be interesting to see if intermittent escalators are willing to continue taking drug despite negative consequences such as shock paired drug infusions. It would also be worthwhile to know if the animals that become escalators have characteristics traditionally considered predictive of addiction vulnerability, such as high impulsivity. There are some small indications of inherent *a priori* differences between the animals that go on to become escalators and those that become non-escalators. For example, it appears that in response to an acute cocaine injection (the initial locomotor test, see Fig. 2.4D), the escalators have a lower locomotion response. A closer examination of the time course of this effect (Fig. 2.4E) indicates that they have a very similar immediate response to the cocaine as the non-escalators, but they seem to have a lower locomotion response in the second half of the session, perhaps indicating an acute rebound effect. In line with this question, it will be important going forward to determine how exactly intermittent access is producing its variability. It might be that there is a special property of the loading phase that causes the transition from recreational use to compulsive use and so the increased maintenance phase prevalent in continuous access is relatively unimportant. It is also possible that it has less to do with the repetitive "loading" *per se* and more to do with the repetitive coming down. Perhaps the animals are undergoing repetitive acute withdrawal stresses from the

intermittent access periods and these cumulative stressors are propelling the behavioral separation.

In chapter 2, I also found that self-administration pattern does affect the characteristics of intracellular calcium waves in the dorsomedial striatum. These calcium events are likely due to intracellular calcium stores and not influx of extracellular calcium (Osanai et al., 2011; Osanai et al., 2006). Given the importance of intracellular calcium signaling to the rewarding and sensitizing aspects of drug use, we thought it likely that escalators would have significantly different calcium waves compared to non-escalators (Barr et al., 2015; Mizuno et al., 2013; Yasui and Su, 2016). Instead, I found that the largest differences were between the continuous access animals and the escalators, perhaps indicating that the differences were due to cocaine intake as continuous access animals took the most drug. The most important subregion of striatum implicated in addiction is believed to change over time (Everitt et al., 2008). It is possible that we would have seen a difference between escalators and non-escalators in the ventral striatum or the dorsolateral striatum.

In chapter 3, I show that PT-type neurons in the ACC contribute to the positive aspects of cocaine experience and IT-type neurons in the ACC contribute to the negative aspects of cocaine experience. Inhibition of PT-type neurons enhanced cocaine reward in a CPP test, while inhibition of IT-type neurons had no effect on cocaine reward. Further investigation of this PT-mediated effect revealed that PT inhibition does not affect natural rewards, nor is it inherently rewarding, but PT neurons are capable of bidirectionally modulating cocaine reward. In contrast, IT inhibition

significantly blunted the aversive properties of cocaine on a conditioned taste aversion task while PT inhibition had no effect.

The ACC is well positioned to process both positive and negative stimuli. It is activated by remote fear memories and chronic pain (Johansen and Fields, 2004; Navratilova et al., 2015; Gao et al., 2017; Frankland et al., 2004). In humans, changes in the ACC have been associated with PTSD, depression, fear, and disgust (Shin et al., 2001; Woodward et al., 2006; Cotter et al., 2001; Auer et al., 2000; Milad et al., 2007; Amir et al., 2005). On the other hand it is also active in response to sexual behavior as well as cocaine and cocaine cues (Frohmader et al., 2010; McLaughlin & See, 2003; Zahm et al., 2010; Neisewander et al., 2010; Zhou et al., 2014). Much like midbrain dopamine neurons, the ACC encodes reward prediction errors as well as reward magnitude and reward probability (Rushworth and Behrens, 2008). Intriguingly, reward prediction errors in the ACC differ from dopamine neurons in one important respect: dopamine neurons encode both positive and negative reward prediction errors, while the ACC is made up of two cell populations, one that encodes positive reward prediction errors and one that encodes negative reward prediction errors (Rushworth and Behrens, 2008). The work in this chapter, though, is the first work to ever show a role for IT and PT neurons in drug addiction. It is also the first work to show that IT and PT neurons in the ACC separately process positive and negative experiences and have opposing roles in addiction. The ACC is an understudied region in drug addiction and this work emphasizes the importance of increased attention towards it going forward.

The inherent aversive properties of drugs of abuse is also an appealing future direction of study. Although there is some research investigating compulsive drug use

that is resistant to negative consequences, there is relatively little work examining the inherent negative properties of drugs and how they contribution to addiction. There is evidence that differential experience of the aversive properties of cocaine predict which animals fail to obtain self-administration (Rademacher et al., 2010). Together with the work in chapter 3, this suggests that IT neurons in ACC may play an important role in the initial acquisition of drug-taking behavior. Further investigation into this connection is an exciting avenue for future research.

In chapter 4, we built on the finding in chapter 3 that PT inhibition was capable of robustly enhancing cocaine reward, by examining if it could also enhance locomotor sensitization, motivation for drug, and drug-seeking. We found that PT inhibition transiently enhanced locomotor sensitization, but the enhancement did not persist to challenge two weeks later. Additionally, PT inhibition did not affect the motivation to obtain drug or drug-seeking during cocaine or cue-induced reinstatement. Finally, we showed that our chemogenetic modulation was effective as $hM4D_i$-mediated inhibition significantly reduced the amount of c-Fos in the ACC following a cocaine reinstatement test.

The differential contribution of PT-type neurons in the ACC during different phases of drug use is an interesting finding. PT neurons were found to play an important role in mediating the positive aspects of the cocaine in a CPP paradigm, but not the negative aspects in chapter 3. It was also found to enhance cocaine-induced locomotor sensitization without altering the motivation to obtain the drug or drug-seeking induced by cue or a drug prime. There are several potential explanations for this dichotomy in results. It is possible that compulsive drug use has little to do with the

rewarding aspects of the drug and that the motivational aspects of drug-craving and drug-seeking are dissociable from the hedonic properties of drugs of abuse. Although motivation and reward are normally considered linked psychological constructs that are inseparable under normal circumstances, there is substantial evidence that the two are dissociable (Robinson and Berridge, 2000). It is possible that PT-type neurons in ACC are therefore responsible for changes in circuits related to locomotor sensitization and drug reward without affecting those parts of the circuitry involved in motivation and drug-seeking, which would drive behavior during a progressive ratio and reinstatement test. It is also possible that at this stage of prolonged self-administration, these tests are driven more by alterations in the valuation of negative components of the drug. There is also substantial evidence that the negative features of drug use are major driving factors in the motivation to obtain drug and the susceptibility to relapse (Koob and Le Moal, 2001). If this is true, then it is plausible that IT-type neurons and not PT-type neurons may be more important for driving behavior on progressive ratio and reinstatement tests. It is also possible that much like the striatum, the site of importance in the PFC shifts at different stages of addiction. Over time the region of importance is believed to shift from ventral striatum to dorsomedial striatum and ultimately to dorsolateral striatum (Everitt et al., 2008). It is possible that ACC is important during a different stage of addiction then we tested here.

The intermittent access model was chosen because it has shown itself to be adept at modeling addiction and inducing addictive behaviors (Zimmer et al., 2012; Calipari et al., 2013; see chapter 2). The PFC becomes increasingly hypoactive with compulsive drug use in humans and rodents (Chen et al., 2013; Dackis & O'Brien,

2005; Volkow et al., 2004). It is possible then, that our inhibition of PT cortical neurons during the progressive ratio and reinstatement tests was without effect due to reaching a floor effect. If the PFC becomes hypoactive with compulsive drug use, it is possible that PFC activity, including PT activity, was already depressed following the intermittent access self-administration paradigm and could not be attenuated far enough to drive a behavioral change. This seems unlikely given our c-Fos results; however, it does remain possible that the reduction in c-Fos after cocaine-induced reinstatement was insufficient to produce a change in behavior.

In the future, this hypoactivity hypothesis could be tested with either *in vivo* electrophysiology or calcium imaging to see how these cortical neuronal populations act throughout self-administration behavior. In conjunction with this, it would be worthwhile to determine if a restoration of glutamatergic tone from the PT-type neurons would be sufficient to reduce responding during progressive ratio and reinstatement tests. Finally, it is quite possible that PT inhibition during the acquisition and initial drug use in self-administration would have yielded an effect on the progressive ratio and reinstatement tests as the enhanced reward during initial use could have created long-lasting reinforced changes that could have driven future likelihood to seek and obtain drug. Attempting to attenuate PT neuron activity after the end of self-administration may have been too late to have an effect.

In conclusion, this thesis provides evidence for the validity of an intermittent access self-administration model in studying individual differences in addiction behavior. It also provides new evidence for the functional separation of IT and PT neurons in ACC in drug addiction. This work opens up appealing new avenues in behavioral modeling of

drug addiction and offers new insight into our understanding of corticostriatal circuits in addiction.

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Education

University of Washington, Seattle, WA
PhD Program in Neuroscience
Supervisor: Susan Ferguson, PhD

University of Michigan, Ann Arbor, MI
BS in Brain, Behavior, and Cognitive Science with High Honors and Highest Distinction, 2011
Concentration GPA: 4.00/4.00; Overall GPA: 3.95/4.00

Honors and Awards

Michigan Promise Award, 2007
University of Michigan Regents Merit Scholarship, 2007
University of Michigan Scholar Recognition Award, 2007
University Honors, 2008-2010
University of Michigan James B. Angell Scholar, 2009-2011
Member of Phi Kappa Phi, 2010
Member of Phi Beta Kappa, 2011
University of Washington Graduate Opportunity Research Assistantship Award, 2012
Achievement Rewards for College Scientists Fellowship, 2012
Ford Foundation Fellowship Honorable Mention: 2014
University of Washington NIDA Institutional Predoctoral Training Grant: 2014-2015
University of Washington Graduate School Fund for Excellence and Innovation (GSFEI) Travel Award: 2016

Peer-reviewed Publications

Yager LM, **Garcia AF**, Donckels E, Ferguson SM. Chemogenetic inhibition of direct pathway striatal neurons normalizes pathological, cue-induced reinstatement of drug-seeking. *Addiction Biology*. Accepted. PMID: 29314464

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,* 16, 30278-7. PMID: 28552825

Yager LM, **Garcia AF**, Wunsch AM & Ferguson SM. (2015). The ins and outs of the striatum: Role in drug addiction. *Neuroscience*, 301, 529-541. PMID: 26116518

Abstracts

Garcia AF, Webb IG & Ferguson SM. Role of PT-type cortical neurons in cocaine-mediated sensitization, conditioned place preference, and reinstatement (2017). Society for Neuroscience, Washington, D.C.

Garcia AF, Yager LM & Ferguson SM. Effect of cocaine self-administration pattern on reinstatement, sensitization, and striatal activity (2017). Winter Conference on Brain Research, Big Sky, MT.

Garcia AF, Yager LM & Ferguson SM. Effect of cocaine self-administration pattern on reinstatement and corticostriatal activity (2016). Winter Conference on Brain Research, Breckenridge, CO.

Garcia AF, Yager LM & Ferguson SM. Cocaine-induced alterations in calcium signaling in the striatum (2015). Winter Conference on Brain Research, Big Sky, MT.

Williams CA, **Garcia AF**, & Perkel DJ. Calcitonin gene related peptide (CGRP) increases neuronal activity in the zebra finch premotor song nucleus RA (2014). Society for Neuroscience, Washington, D.C.

Talks

Weill Cornell Medicine, 2018
Icahn School of Medicine at Mount Sinai, 2018
University of Minnesota, 2018
Winter Conference on Brain Research, 2018
Achievement Rewards for College Scientists (ARCS) luncheon, 2015

Teaching

Mentored four undergraduate research assistants and one high school student, 2013-2017
Teaching Assistant: “Introduction to Systems and Behavioral Neurobiology”, 2014
Guest Speaker: “Preparing for Graduate Education”, 2014

Outreach

Volunteer at Science Adventure Lab field trips with under-sourced elementary schools, 2016-present
Volunteer at Brain Awareness Week activities hosting local schools, 2015-2017
Volunteer at Seattle Children’s Science Block Party, 2016
Performed classroom visit at Bear Creek Elementary, 2016
Performed classroom visit at Bozeman High School, 2015, 2017

Performed classroom visit at Upper Blue Elementary, 2016
Volunteer at Mountain Top Children's Museum, 2016