Role of the Ventral Striatal Pathways in Reinforcement Learning: Chemogenetic Modulation During the Incubation of Craving

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Abstract

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Cocaine addiction is a major public health problem with high rates of relapse. The incubation of cocaine craving, characterized by an increase in cue-induced cocaine seeking behavior after abstinence, is thought to play a key role in relapse. Previous research has suggested that the ventral striatum, particularly the nucleus accumbens (NAc), is critical for the expression of cocaine-seeking behavior. Inhibition of adenylate cyclase by G_i-coupled receptors is a key mechanism that modulates the activity of direct pathway (D_1 receptor expressing) and indirect (A_{2A} receptor expressing) medium spiny neurons. Furthermore, the serotonin 5- receptor (a G_i-coupled receptor has been implicated in the regulation of cocaine-seeking behavior in these neurons. However, the specific role of 5- receptors in the NAc following the incubation of cocaine craving remains unclear. Therefore, we explored the role of G_i-mediated signaling in the incubation of cocaine craving in two ways—by stimulating transgenic hM_4Di DREADD receptors selectively expressed in direct or indirect pathway medium spiny neurons immediately before testing for the incubation of craving and by measuring 5- mRNA levels

immediately after testing. We used 6- and 3- hour access self-administration sessions, within-session threshold procedures, and Cre-dependent viral expression of RiboTag and hM_4Di in D_1-Cre and A_{2a}-Cre transgenic rats to assess differences between the direct or indirect pathways in the NAc. After a period of forced abstinence, we assessed the incubation of cocaine craving by measuring cue-induced cocaine seeking behavior. To examine the role of 5- receptors in the NAc, we used RiboTag RNA immunoprecipitation to selectively isolate mRNA from D_1 and A_{2a} receptor-expressing neurons in the NAc. We then used qPCR to measure mRNA levels of 5- receptors in these neurons following the incubation of cocaine craving. Our findings suggest that activating Gi-signaling in A_{2a}-expressing indirect pathway medium spiny neurons during the incubation of cocaine craving reduced cue-induced cocaine seeking. The finding that activating Gi-signaling in indirect pathway medium spiny neurons reduces the incubation of craving is a surprising result and illustrates the complex role that the output pathways from NAc regulate drug seeking. While, our preliminary data did not find significant changes in 5- receptor expression in either D1 or A2a receptor-expressing neurons in the NAc, serotonin action at these receptors is still likely to be an important modulator of drug seeking. However, our findings further distinguish the nuances of 5- receptor activation in the processing of stress, reward processing, and relapse; additionally, this study suggests unique functional roles of the ventral striatal pathways following prolonged abstinence.

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CHAPTER 1. INTRODUCTION

1.1 OVERVIEW: THE INCUBATION OF COCAINE CRAVING

Cocaine addiction is a debilitating neuropsychiatric disorder. Patterns of cocaine use shifted in several ways from the 1990s through 2020. In the 1990s, cocaine use, particularly crack cocaine, was at its peak in the United States. The use of crack cocaine was especially high in urban areas and was associated with a surge in drug-related violence and crime. However, since the late 1990s, overall cocaine use has declined. Cocaine use has historically affected marginalized populations more severely: cocaine use was more prevalent among certain racial and ethnic groups, with higher rates among non-Hispanic Whites and Hispanics compared to non-Hispanic Blacks. Individuals diagnosed with cocaine addiction or dependence often entered treatment programs (Mustaquim et al., 2021). The Treatment Episode Data Set provides information on the demographic and substance use characteristics of people admitted to substance abuse treatment facilities. Data from the early 2000s showed that around 14% of treatment admissions were for cocaine abuse, with most of those admissions for smoked cocaine (SAMHSA, 2022). Treatment effectiveness varies depending on the individual, the severity of the addiction, and the treatment program itself. There is no single treatment that is effective for everyone. Cocaine addiction treatments generally consist of behavioral therapies, such as cognitive-behavioral therapy, contingency management, and the community reinforcement approach. While there are no FDA-approved medications specifically for cocaine addiction, ongoing research is exploring the development of medications that target the neurobiological processes underlying cocaine addiction. It is important to note that treatment outcomes depend on various factors, including the quality and duration of

treatment, the individual's motivation to change, and the availability of aftercare support. Studies have shown that participation in long-term treatment programs and the use of evidence-based therapies can lead to better outcomes for individuals with cocaine addiction. Some pharmacological interventions are shown to increase retention in treatment and decrease relapse risk by mitigating cravings (Saunders et al., 2015). Understanding the cellular and molecular mechanisms underlying the sex- and genotype-specific effects on drug-seeking behavior is essential for the development of targeted therapeutic interventions.

The intention of this introductory chapter is to present a basic overview of the underlying themes that tie the subsequent experimental chapters together. Although the experiments within each chapter differ in many ways due to differences in the behavioral approach, brain regions, and species, the underlying neurocircuitry has interconnecting loops that indirectly affect the overall system. It is important to consider the implications of each experiment within the broader context of the signaling pathways, anatomically specific projections, and heterogeneous molecular profiles within the various types of neurons, interneurons, and glial cells. Of the many innovative techniques developed recently in Neurosciences, my dissertation utilized a combination of rodent behavioral models of addiction, RiboTag cell type specific mRNA purification, used anatomical targeting strategies to enhance our chemogenetic precision, and employed cell-type specific viral-mediate gene expression to investigate differences and similarities between the two major midbrain striatal pathways.

1.2 Behavioral Models of Addiction

Self-administration models of addiction have significantly contributed to our understanding of the neurobiology of addiction by demonstrating the importance of volitional drug use, or the act of consciously and intentionally consuming a drug. This paradigm, which mimics the human condition of drug addiction more closely than non-volitional drug administration, has been critical in recognizing the fundamental roles of choice, reinforcement, and motivation in the development and maintenance of drug addiction. When drugs are self-administered, individuals can control their intake based on various factors such as desired effect, tolerance, withdrawal, and body weight, much like in the human condition (Nair et al., 2009; Nair et al., 2008). This aspect of self-dosing allows researchers to observe the complex relationship between escalating drug use and the subsequent adaptations that occur in the brain, which might not be as evident in forced noncontingent models (Kawa et al., 2016). The stress of noncontingent drug-exposure influences pharmacology and stress is also a well-documented trigger for drug craving and relapse (Zorrilla et al., 2014). When drugs are forcibly administered, it introduces a level of stress that may confound the effects of the drug itself, altering the neurochemical response. This is important as stress and negative affect are considered key factors in the transition from casual to compulsive drug use and relapse (Nair et al., 2011; Ramchandani et al., 2018). In addition, studies involving self-administration show how drugs of abuse can hijack the brain's natural reward system, conditioning the individual to seek out the drug despite negative consequences, a hallmark of addiction. Over time, the brain adapts to the drug, resulting in tolerance and withdrawal symptoms, pushing the individual to increase their intake (Uhl et al., 2019). These models have significantly improved our understanding of the complex interplay of factors including

dose, body weight, and tolerance, consumption frequency and relapse related to addiction and informs the development of better prevention and treatment strategies.

1.2.i Conditioned Place Preference

Although it is not technically a self-administration model, conditioned place preference (CPP) is a useful tool for using A/B testing to compare context associated preferences. CPP compares the relative value between two rewards by pairing them with distinct contextual cues (i.e., Drug A in Context A and Drug B in Context B). The rat is conditioned to each chamber with each reinforcement treatment. A probe test is then given where the subject is allowed to freely move between the two contexts; the ratio of time spent in each context versus the neutral zone provides a metric of a drug's rewarding value. An alternative approach would compare the rewarding value of a drug against the rewarding value of that drug following a pharmacological, chemogenetic or optogenetic intervention. This model has implicated many brain regions within the limbic system, prefrontal cortex, and midbrain structures in the learned associations between contextual cues and drug experiences, allowing us to infer the rewarding properties of a drug. Of particular importance, the dorsal and ventral striatal pathways have a demonstrated role in CPP (Ferguson et al., 2008; Neumaier et al., 2002; Schindler et al., 2012).

1.2.ii Stages of Addiction Cycle and Various Self-Administration models

Of the roughly 25% of individuals that develop addiction-like behaviors, the timeline when symptoms worsen can vary considerably. Addiction can be broken down into major stages including the acquisition, maintenance, escalation, and early withdrawal, prolonged abstinence, and the incubation of drug craving, as well as extinction and

reinstatement. Each period has unique patterns of gene expression, pathway signaling, and neuroanatomy that regulate its severity. Self-administration models are useful methods for investigating these issues. Rodents are trained to perform an operant response, such as lever pressing or nose poking, to receive a drug infusion directly into their bloodstream via intravenous catheter implant. Before the self-administration training, rats must undergo surgery to implant an indwelling catheter into a major vein, usually the jugular vein. Following surgery and recovery, rats are placed in an operant conditioning chamber equipped with levers or nose poke holes, stimulus lights, and a drug infusion system. The catheter is securely connected to a swivel system that allows the animal to move freely in its experimental chamber. The training phase begins with a "priming/shaping" training session, where the rat learns to associate the operant response (lever press or nose poke) with the reinforcer (typically food or drug). For drug self-administration, the operant response is linked to the drug infusion, while the inactive response results in no consequence. Once the rats acquire the operant response and reliably self-administer the drug, the maintenance phase begins. This phase can be broadly implemented with distinct effects on behavior through manipulation of the reinforcement schedule, reinforcer value, and selection of withdrawal parameters and reinstatement probe tests (Koya et al., 2009; Li & Frantz, 2009; Neumaier et al., 2009). Acquisition could be considered the initial exposure to a drug-reinforcer or the reintroduction of a drug-reinforcer following extinction or abstinence. Once acquired, the maintenance stage begins after an individual's consumption remains consistent over a set number of days, suggesting their preferred pattern and level of drug consumption. Like the acquisition phase, the maintenance phase can be used as retraining and be reimplemented between probe/reinstatement tests for within-subject comparisons.

Escalation refers to an increase in maintenance responding over time and is usually only reported in rats that have long access sessions or intermittent drug availability (Eskenazi & Neumaier, 2011). Briefly, prolonged abstinence is any forced period of sobriety where no drug context or drug-related cues are given (Grimm et al., 2003; Neisewander et al., 2014). This leads to the incubation of craving as described above. Traditionally, the propensity to relapse to drug seeking is explored using extinction learning models, where the animal no long receives drug delivery or conditioned cues after responding; reinstatement of drug seeking after extinction training can be assessed by reintroducing drug-related cues, by exposing the subject to a stressor by administering a small dose of drug (Grimm et al., 2003; Koya et al., 2009). The model used in this dissertation research, incubation of craving, involves a period of forced abstinence after drug self-administration, with no extinction sessions, followed by a probe test for drug seeking; this model is sensitive to some important adaptation that proceed after active drug taking has stopped (Chiu et al., 2021; Szumlinski & Shin, 2018). Together, these procedures constitute some of the common behavioral self-administration paradigms that reveal important aspects of the different stages in the progression of addiction, but there are others which allow measuring the motivation to seek a drug, the reinforcing value of a drug, and the elasticity of drug demand in more nuanced ways (Oleson & Roberts, 2019). Incorporating behavioral economics into research on addiction and reinforcement learning will be discussed in a later section.

1.2.iii Factors that Influence the Progression of Addiction Severity

The schedule of reinforcement, session duration, and drug doses influence the outcome of various aspects of drug-taking behavior. Common schedules of reinforcement include

fixed-ratio (FR), variable-ratio (VR), and progressive-ratio (PR) schedules. FR schedules require the rat to perform a fixed number of responses to receive a single drug infusion, e.g., FR1 means one response per infusion and FR5 means five responses per infusion (Pentkowski et al., 2014). This schedule measures the rate of drug intake and response patterns. VR schedules involve a variable number of responses per drug infusion, averaging to a predetermined ratio. This schedule leads to a high and stable rate of response and is often used to study drug-seeking behavior and resistance to extinction (Garcia et al., 2017). PR schedules involve an increasing number of responses required for each subsequent drug infusion. The "breakpoint", or the highest ratio completed before the rat stops responding, is a measure of the motivation to take the drug (Brodsky et al., 2016). The self-administration sessions can continue for several days or weeks to study the acquisition, maintenance, and escalation of drug intake. Following self-administration, rats can undergo extinction training, where drug infusions are no longer available, and the rats learn that the operant response no longer results in drug delivery. After extinction, researchers can examine drug or cue-induced reinstatement of drug-seeking behavior to model relapse in addiction (Nair et al., 2009; Nair et al., 2013). The retesting of animals following retraining is an excellent way to increase statistical power and reduce the number of rodents needing to be sacrificed.

1.3 RiboTag & RNA Sequencing: Revolutionizing Science

The RiboTag system works by genetically altering Rpl22, a gene that encodes a ribosomal protein involved in protein translation by tagging this molecule with hemagglutinin (HA), a small epitope that allows efficient binding by commercially available antibodies (Sanz et al., 2009). Using anti-HA antibodies conjugated to magnetic beads, the ribosomal-

associated mRNA that are actively undergoing translation can be captured and immunopurified from cells that express HA-tagged Rpl22 protein (i.e. “RiboTag”). One way to accomplish this is to use a transgenic mice model where RiboTag is expressed in a Cre-dependent manner. Cre recombinase expression can be controlled using tissue- or cell-specific promoters, or by injecting AAV-Cre into specific brain regions of interest in the rat. Brain tissue from the region of interest is homogenized in a buffer containing RNase inhibitors and cycloheximide, which locks RNAs that are actively undergoing translation onto their associated ribosomes (Lesiak et al., 2015). This breaks down the cells and releases the cellular contents, including the ribosomes and bound mRNA into the supernatant. By adding the anti-HA antibody beads, RiboTag-containing ribosomes can be bound whereas ribosomes from cells that do not express RiboTag are not bound by the anti-HA antibody beads. After washing to remove non-specifically bound material, the HA-tagged ribosomes and associated mRNAs can be eluted from the beads. The mRNA can then be purified using standard Qiagen Kit (Lesiak & Neumaier, 2016). The isolated mRNA can be analyzed to explore genes being actively translated in the cell type of interest; these techniques include qRT-PCR or RNA sequencing (RNASeq).

The advent and implementation of RNASeq technology has ushered in a new era of neurobiological research, enabling unprecedented precision in elucidating cell-type specific gene expression patterns across the brain. The power of RNASeq lies in its ability to reveal the complex and diverse transcriptomic landscape of the brain, providing detailed insights into the unique gene expression profiles that define specific neuronal subtypes (Levinstein et al., 2020). The spatial resolution afforded by this technology is transformative, enabling a robust characterization of the topography of gene expression

across the brain. This level of detail allows researchers to elucidate the intricate interplay between different brain regions, as well as to discern how specific neuronal subtypes contribute to overall brain function and behavior. Advances in this field are not only refining our understanding of the brain's functional architecture but also paving the way for innovative treatment strategies by allowing high precision readouts of the translatome at the resolution of a single neuron (Gao & Zhao, 2021). This includes the potential for personalized medicine that considers the unique genetic and molecular signatures of individual patients. Furthermore, by providing a deeper understanding of neurocircuitry and its interactions, we can identify novel therapeutic targets that can be modulated to treat various neurological and psychiatric disorders (Lesiak et al., 2021). This new perspective in understanding the cellular heterogeneity within the brain could potentially unlock doors to creating highly targeted therapies, improve our understanding of disease onset and progression, and enable personalized medicine based on the patient's unique molecular makeup. It is an exciting time for neurobiology, and RNASeq technology propels the field past many of the limitations found in modern techniques. The potential for error can be mitigated by ensuing that the samples meet a certain quality threshold before sending samples off to be sequenced (Kronman et al., 2019).

The first step in RNA-Seq is to isolate total RNA from your cells or tissue of interest. This is typically achieved by using a variety of commercially available kits or traditional methods like phenol-chloroform extraction. The quality and integrity of the RNA are important and should be checked prior to proceeding to the next step. The isolated RNA is then converted into a cDNA library. In most cases, mRNA is the RNA type of interest. Therefore, the RNA sample often undergoes a poly-A selection step to select for mRNA

molecules, which have a poly-A tail. Alternatively, ribosomal RNA can be removed since it represents a significant proportion of total RNA but is usually not of interest. The selected RNA is then reverse transcribed into cDNA. The cDNA is then fragmented, and adapters are ligated to the ends of these fragments. These adapters are necessary for the cDNA to bind to the sequencing platform. The library is then sequenced using one of several available sequencing technologies such as Illumina's sequencing-by-synthesis technology. Sequencing can be done in either single-end or paired-end mode. Single-end reads only sequence one end of each cDNA fragment, while paired-end reads sequence both ends. Paired-end sequencing can provide more information, such as the detection of fusion transcripts, but it is also more expensive (Coffey et al., 2022; Lesiak et al., 2021). The whole RNA-Seq process, from sample preparation to data analysis, requires not only a good understanding of molecular biology but also a reasonable level of bioinformatics skills to process the large amount of data produced. It's also worth noting that there are other considerations when planning an RNA-Seq experiment, such as the depth of sequencing required (how many reads you need to sufficiently cover your transcriptome) and biological/technical replicates to ensure your results are reliable and reproducible.

1.4 Monoamine Projections Across the Striatal Circuitry

Monoamines are a class of neurotransmitters that include dopamine (DA), norepinephrine, and serotonin (5-HT) which originate from the ventral tegmental area (VTA), locus coeruleus, and raphe nucleus, respectively. Cocaine’s mechanism of action targets monoamine transporters including in terminals of the striatum. These pathways represent complex circuits that, through their interactions, modulate a wide range of behaviors and functions, including motor control, reward, and aversion. Their intricate

interplay is crucial for normal function and disruptions in these circuits are associated with various neuropsychiatric conditions, including Parkinson's disease, addiction, and depression. Table 1.1a Afferents and Efferents of Ventral Striatum describes the integrated inputs and outputs of this system.

Table 1.1a Afferents and Efferents of Ventral Striatum

Brain Region	Input	Output
NAc (shell and core)	- Dopaminergic inputs from VTA - Glutamatergic inputs from various cortical and limbic regions	- GABAergic output to VP and SN
VTA	- Inputs from LHb (GABAergic)	- Dopaminergic output to NAc and other brain regions like the prefrontal cortex
SN	- Inputs from various brain regions (including VTA)	- Dopaminergic output to various brain regions
VP/GPi	- GABAergic inputs from NAc	- Outputs to the thalamus and LHb
LHb	- Inputs from VP and NAc	- GABAergic inhibitory projections to VTA and SN - Glutamatergic excitatory projections to DRN and RMTg
DRN and RMTg	- Excitatory inputs from LHb	- DRN: Serotonergic neurons regulated by LHb, RMTg, elsewhere - RMTg: GABAergic output to VTA

ABBREVIATIONS

NAc: Nucleus accumbens
VTA: Ventral tegmental area
SN: Substantia nigra
GPi: Globus Pallidus interna
LHb: Lateral habenula
DRN: Dorsal raphe nucleus
VP: Ventral pallidum
RMTg: Rostral medial tegmental nucleus

1.4.i Lateral Habenula Output Projections

The lateral habenula (LHb) sends inhibitory projections to the VTA and DRN. The LHb-VTA pathway is believed to modulate dopamine release, while the LHb-DRN pathway influences serotonergic activity. Both pathways play crucial roles in regulating reward and aversion behaviors. Interestingly, the LHb also communicates with the rostromedial-tegmental nucleus (RMTg), which is considered as a 'stop' signal hub for dopaminergic activity in the VTA but also sends inhibitory projections to DRN. In addition to the projections to the VTA and dorsal raphe nucleus (DRN), the LHb also sends inhibitory projections to other areas including the GABAergic RMTg. It projects to various other areas involved in reward processing and aversive responses, such as the hypothalamus and the basal ganglia.

1.4.ii Direct and Indirect Output Projections of the Nucleus Accumbens

Within the NAc, D1-receptor expressing medium spiny neurons (MSNs) send projections directly to dopaminergic nuclei, primarily to the VTA and subtantia nigra (SN), another region involved in reward and movement. D1 MSNs are typically associated with the 'direct' pathway facilitating movement, while D2 MSNs contribute to the 'indirect' pathway inhibiting movement. This connection forms a key part of the mesolimbic pathway, which is involved in reward and reinforcement. The NAc is anatomically subdivided into two adjacent regions, a core surrounding the anterior commissure and the shell outlining the core along the laterally and ventrally. The shell has been shown to project almost exclusively to the VTA while the core has been shown to project to the SN. Their differences will be discussed further in a later chapter (Smith et al., 2013). Briefly, the direct and indirect pathway projections allow the NAc to fine-tune the direction and

strength of its downstream signaling. Considering cell-type specificity based on molecular profiles, D1-expressing MSNs primarily terminate onto the SN yet an emerging portion terminate onto the VP. At least for the ventral striatum, D2-expressing MSNs selectively project to the VP; D2 MSNs are rarely, if ever, observed projecting to the VTA/SN. These distinct pathways operate together to enable smooth and coordinated movements. In essence, the D1 and D2/A2a-expressing MSNs in the NAc that project to the VP and VTA form interwoven circuits that work together to regulate reward and movement. Despite their heterogeneous distribution across the NAc and their individual roles, these circuits function in tandem to maintain balance within the basal ganglia.

1.4.iii VTA Outputs and Primary Synthesis of Dopamine

The VTA is the origin of the dopaminergic cell bodies of the mesocorticolimbic dopamine system and one of the dopamine pathways in the brain. It is widely implicated in drug as well as natural reward circuitry and is vital to the function of the brain's reinforcement valuation system. The VTA neurons synthesize dopamine via an enzyme called tyrosine hydroxylase (TH). TH is the rate-limiting enzyme in the synthesis of other catecholamines. It works by converting the amino acid tyrosine into a molecule called L-3,4-dihydroxyphenylalanine, which is then further converted into dopamine by the enzyme DOPA decarboxylase. Because TH is the first enzyme in this pathway and is not saturated with its substrate under normal conditions, it serves as the primary regulatory point in dopamine synthesis. The activity of TH can be increased or decreased in response to inputs it receives from the NAc core, NAc, shell, LHb, or other signals, thus controlling the production of dopamine. Furthermore, the VTA sends dopaminergic projections to various regions throughout the brain. The reciprocal projections to the NAc are the

primary component of the reward pathway and are involved in encoding positive reinforcement and reward expectation. Projections to the prefrontal cortex are involved in aspects of executive function such as decision-making. The VTA to amygdala projection is involved in processing emotional reactions and fear conditioning. Meanwhile, hippocampal projections play a role in memory formation and consolidation. While traditionally the cerebellum has been considered as a motor structure with little direct connection to the dopaminergic system, there are studies suggesting dopaminergic projections from the VTA to the cerebellum. However, it should be noted that these connections are not as well-studied (Anderson et al., 2003; Anderson et al., 2006).

The connections between these brain structures involve several neurotransmitters, such as dopamine, GABA, and glutamate. Dopaminergic projections from the VTA to the NAc play a significant role in reward processing, while GABAergic and glutamatergic connections within the circuit regulate the activity of these regions. The lateral habenula serves as a crucial hub in this network, influencing both dopaminergic and serotonergic systems through its projections to the VTA, SN, DRN, and RMTg. Studies have reported the LHb having bidirectional outputs (via GABA and Glutamate corelease). A secondary feedback loop is in place compliments of the the GABAergic interneurons in the VTA. A greater inhibition effect is possible by amplifying the signal via glutamatergic signal from the LHb through the RMTg onto the inhibitory interneurons within the VTA that project back to the VP and NAc (which is another opportunity to finetune the level of dopamine via the reciprocal innervation of the NAc and VP).

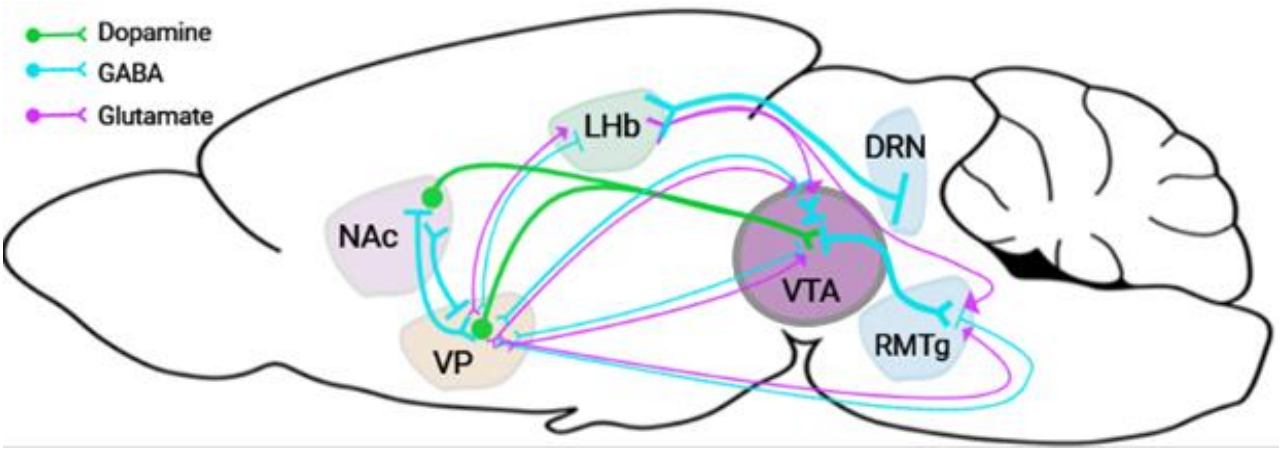

*Figure **1.4**.A Lateral Habenula Bi-Directional Control of Dopamine Signaling*

1.5 Chapter 1 References

Chapter 1

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CHAPTER 2. 5HT1B IN THE MOUSE VENTRAL STRIATUM

An appropriate amount of rephrasing, reformatting, and reconceptualizing was performed on a previously peer-reviewed publication to allow inclusion in this dissertation. The manuscript was published by Addiction Biology. Sunila Nair was the first author; I co-authored by aiding in editing, experimental design, and execution. The full citation is:

(Fontaine et al., 2022) Fontaine, H. M., Silva, P. R., Neiswanger, C., Tran, R., Abraham, A. D., Land, B. B., Neumaier, J. F., & Chavkin, C. (2022). Stress decreases serotonin tone in the nucleus accumbens in male mice to promote aversion and potentiate cocaine preference via decreased stimulation of 5-HT(1B) receptors. Neuropsychopharmacology, 47(4), 891-901. https://doi.org/10.1038/s41386-021-01178-0

2.1 INTRODUCTION

5-HT$_{1B}$ receptors are one of 14 serotonin (5-HT$_{1B}$) receptor subtypes and belong to the G-protein coupled coupled receptor (GPCR) family. When activated by serotonin, these receptors initiate a signaling cascade involving G-proteins and downstream effectors, such as ERK1 and ERK 2, MAP Kinase (MAPK), and Beta-Arrestin (Bruchas et al., 2011; Liu et al., 2019; Pullarkat et al., 1998). By phosphorylating these proteins, 5-HT$_{1B}$ receptors are able to selectively mark which downstream effectors need to be modulated (Liggett, 2011). Another mechanism of 5-H's selective action within the striatum is due to the different binding affinity of various pharmacological agonists and antagonists on the family of serotonin receptors (Hagan et al., 2011; Murrough, Czermak, et al., 2011; Murrough, Henry, et al., 2011). Advancements in x-ray crystallography have been able to visualize the specific binding sites that allow for increased selectivity across common serotonin receptors(Wacker et al., 2013).

When serotonin binds to the 5-HT$_{1B}$ receptors, it causes a conformational change in the receptor, enabling the activation of the different associated G-proteins. 5-HT$_{1B}$ receptors are primarily coupled to the inhibitory Gαi/o family of G-proteins. Upon activation, the Gαi/o subunit releases a bound GDP molecule and binds to GTP instead. This process

causes the Gαi/o subunit to dissociate from the Gβγ subunits. The Gαi subunit, now bound to GTP, inhibits the activity of the enzyme adenylate cyclase (Liu et al., 2019). This results in a decrease in the conversion of ATP to cyclic AMP (cAMP). The reduction in cAMP levels leads to a decrease in the activation of protein kinase A (PKA). With less active PKA, there is reduced phosphorylation of downstream targets, including ion channels, receptors, and other proteins (Ghavami et al., 1999; Renner et al., 2012). This can affect neuronal excitability, neurotransmitter release, and intracellular signaling pathways (Sari, 2004). It is generally agreed that 5-H serves a complex role in maintaining allostasis within the limbic circuitry systems. Our dissection of the intricacies will be crucial for deepening our understanding of 5-HT_{1B}'s multi-faceted role. Furthermore, this study aims to better understand the roles and interactions of pre-synaptic autoreceptors and post-synaptic heteroreceptors. The exact contribution of 5-H signaling within addiction, learning, and reward processing is critically dependent on the specific timing and topographical positioning within various brain regions. With the extensive variables at play, this project will specifically address assumptions on the effect of quantity of cocaine consumption, pattern of maintenance consumption, potential sex differences, length of withdrawal, brain region dependent effects, and differences in two distinct projectile neurons overlapping within the same subregion. It is important to keep in mind that even localized tissue targeting can still affect multiple distinct neuronal populations with distinct anatomical and molecular profiles, specifically in regions with prominent cellular subtypes like in the direct and indirect nucleus accumbens pathways. The Neumaier Lab has extensively investigated the nuanced interactions of mild stress and

potentiation of a psychomotor amphetamine response, drug valuation and reinforcement strength, as well as withdrawal and abstinence (Neumaier et al., 2002).

2.2 Experiment 1: Assessing 5-HT1B Dynamics in the Direct and Indirect Pathways in a Mouse Stress Model

To further deepen our understanding of cell-type specific differences, we quantified 5-HT_{1B} mRNA expression with in-situ hybridization in the nucleus accumbens. To assess the possibility of 5-HT_{1B} potentiating stress, we performed rFSS and extracted brains 30 minutes and 24 hours later. 5-HT_{1B} receptors are Gi-coupled GPCRs; that inhibit neurotransmitter release and have been implicated in regulating the response to stressors and psychostimulants. Transient antagonism of 5-HT_{1B} receptors in the NAc produced similar increases in cocaine preference. This is possibly a maladaptive coping strategy where increases in drug and social reward seeking attempt to buffer the negative effects such as anhedonia. The data suggests that serotonin signaling produces a feedback system that allows modulation of signaling across proximal and distal pathways by initiating postsynaptic cascades through efferent receptors and self-monitoring with presynaptic terminal auto-receptors. Also, these findings allude to the existence of a generalizable potential pathway that affects other aspects of reward processing, learning, and memory. The increased cocaine preference induced by stress was blocked by additional pharmacological antagonist treatment that blocked 5-HT_{1B} receptors during conditioning. This study promotes further investigation of 5-HT_{1B} on not only initiating reward potentiation, but the lasting mediation of reinforcement learning.

Figure 2.2a demonstrates the ubiquitous expression of Htr1B transcript across the medial NAc. These were not significantly different at baseline between prodynorphin (Pdyn) and

adora2a (A2a) expressing neurons, or ChAt interneurons. Interestingly, there was a fleeting increase in Htr1B transcript levels specifically in Pdyn+ neurons 30 minutes following the stressor, but levels seem to return to baseline by the 24-hour time point. This further supports a precise and coordinated role of 5-HT_{1B} receptor modulation across distinct regions and cell populations. It is not immediately clear why the effect would be limited to the direct pathway or whether the increase would produce functional receptors. The expansive impact that NAc 5-HT_{1B} receptors is having on the regulation of affect and substance use implies there is a fundamental contribution to neuronal and synaptic function. Specifically, there is evidence to suggest that 5-HT_{1B} receptors are intimately involved with providing SERT feedback relating to sensitization, tolerance, withdrawal, and relapse (Neumaier et al., 2002). Much of the progress made over the last decade capitalized on pharmacological techniques with; using strategic combinations of agonists and antagonists overcomes most drawbacks. However, it is extremely rare that a pharmacological tool is fully characterized as being absolutely unique in its action or side effects peripheral systems. Other caveats include discrepancies with pharmacological action and genetic knockout approaches. These discrepancies are artifacts of ambiguity in that they could be attributed to uncontrolled variables or unexpected consequences. Most pharmacology is “sticky” and it should not be too surprising that if you remove a gene from before birth, there could be long-lasting unanticipated developmental consequences that may not manifest in an apparent way.

2.3 MATERIALS AND METHODS

2.3.i Animal Husbandry

Adult (8-20wk) male C57BL/6 mice and transgenic strains on C57BL/6 genetic background were group housed (2-5/cage), given access to food pellets and water ad libitum, and maintained on a 12hr light:dark cycle (lights on at 7AM). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee and conformed to US National Institutes of Health guidelines.

2.3.ii Forced Swim Stress

Mice were subjected to a modified Porsolt forced swim stress (rFSS) as described previously (Sinha, 2008). All swim sessions were conducted in 31±1°C water. On day 1, mice received a 15min initial swim, followed 22hr later by four 6min swims, each separated by 6min. After each swim, mice were removed from the water, towel dried, and returned to their home cage.

2.3.iii RNAscope in situ hybridization

We performed RNA in situ hybridization for Fos, Drd1, and Drd2 mRNAs as described previously (Li et al., 2015). Immediately after dissecting DS tissue for FACS (see FACS section, above), we froze the rest of the rat brain directly in isopentane (kept on dry ice) for 20 s before we transferred the brain in a sealed bag to 80°C for long-term storage. After equilibrating brains in a cryostat (CM 3050S) at -20°C for 2 h, we collected 16 m brain slices at approximately Bregma -0.24 mm (Paxinos & Watson, 2013) and mounted slices directly onto Super Frost Plus slides (Fisher Scientific). We left slides at -20°C for

10 min and then stored them at -80°C until ISH assay. We used RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics) and performed ISH assay according to the user manual for fresh frozen tissue. On the first day, we fixed brain slices in 10% neutral buffered formalin (Fisher Scientific) for 20 min at 4°C. We rinsed the slices three times in PBS and dehydrated the slices in 50, 70, 100, and 100% ethanol. We stored slices in fresh 100% ethanol overnight at -20°C. On the second day, we first dried the slides at room temperature for 10 min. To limit the spreading of solutions, we drew a hydrophobic barrier on slides around brain slices. We then treated the slides with protease solution at room temperature for 20 min and then washed it off. We applied 1X target probes for Fos, Drd1, and Drd2 to the slides and incubated them at 40°C for 2 h in the HybEZ oven. Each RNAscope target probe contains a mixture of 20 ZZ oligonucleotide probes that are bound to the target RNA. Next, we incubated the slides with preamplifier and amplifier probes (AMP1, 40°C for 30 min; AMP2, 40°C for 15 min; AMP3, 40°C for 30 min). We then incubated the slides with fluorescently labeled probes by selecting a specific combination of colors associated with each Last, we incubated sections for 20 s with DAPI.

2.4 Results: rFSS increases 5-HT1B transcript expression in Pdyn+ neurons in the NAc

Chronic exposure to stress increases 5-HT_{1B} transcript in the NAc, which may represent a compensatory increase in reward sensitivity in response to stress. However, regulation by sub-chronic stress exposure, such as the rFSS used in this study, has not been detected. We used RNAscope to evaluate expression of Htr1B in NAc subpopulations. In unstressed mice, Htr1B colocalized with Pdyn, Adorsa2a, and Chat-expressing cells. To assess the effects of rFSS on Htr1B expression, brains were dissected 30 min and 24 h after rFSS and

stained tissue sections were compared to unstressed controls (Figure 2.4.I, top row). This may be attributed to low sensitivity of prior techniques or difficulty assessing cell-type specific changes (Figure 2.4.II, bottom row).

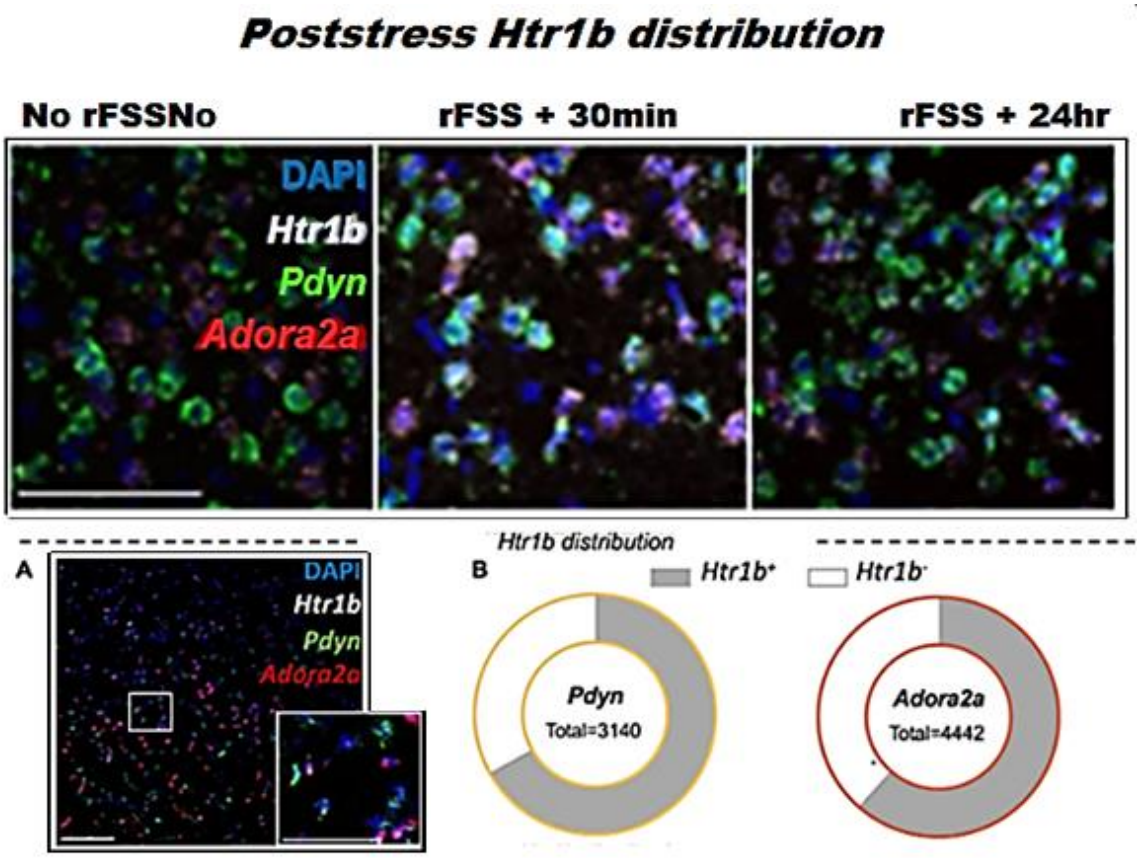
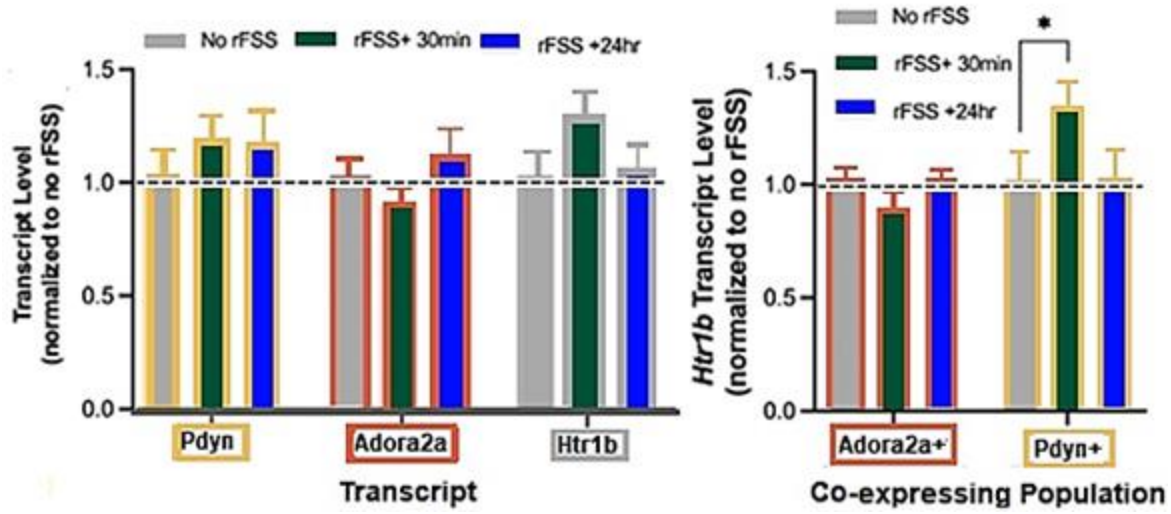


Figure 2.4.I Pdyn, Adora2a, and Htrlb RNAScope

Total levels of Htr1B, Pdyn, and Adora2a transcript did not change after rFSS (two-way ANOVA, $P > 0.05$) (Figure 2.5.III). In contrast, examining the levels of Htr1B in Pdyn and Adora2a subpopulations revealed a significant and selective increase in Htr1B expression in Pdyn+ cells 30 min after stress (two-way ANOVA; 5-HT_{1B} receptors are localized to the terminals of both direct and indirect pathway MSNs, where they inhibit neurotransmitter release into target areas. 5-HT_{1B} receptors are also localized on axon collaterals of MSNs



within NAc. 5-HT$_{1B}$ receptors are also expressed on the terminals of neurons projecting into NAc from DRN (e.g. serotonin neurons), cortex, and amygdala.

Figure 2.4.II Pdyn, Adora2a, and Htrlb Transcript Levels

2.5 CONCLUSIONS

The principal findings of the present study provide insight into the mechanisms by which stress impinges on the serotonin system to sensitize animals to subsequent reward. KOR expression in serotonergic neurons that co-expressed dynorphin in the mNAc played a crucial role in the enhancement of cocaine reward by rFSS. A diagram in Figure 2.5.III provides a visual representation of our findings. They discovered that targeted manipulation of DRN circuitry with precise timing of stimulation specific times indicated that inhibiting serotonin neurons in the DRN can lead to negative emotional responses and increase preference for cocaine. They isolated the effect of decreased serotonin tone at the 5-HT1B receptor by transient blockade of 5-HT1B receptors, which was sufficient to recapitulate the potentiation of cocaine preference observed following KOR activation

(Figure 2.4.II; Figure 2.5.III). Lastly, we found that rFSS selectively increases expression of 5-HT1B transcript in Pdyn+ neurons and not Adora2a+ neurons (Figure 2.4.IFigure 2.4.II). Together, this evidence details a potential Dyn-KOR-5-HT-5-HT_{1B} axis contained within the mNAc, in which rFSS provokes a transient decrease of serotonin tone that is central to passive coping, aversion, and increased cocaine preference.

One potentially important consideration is that 5-HT_{1B} receptor expression is heavily influenced by numerous environmental factors such as exposure to stressors, drug exposure, and novelty of these experiences (Levinstein & Neumaier, 2017; Liu et al., 2019). In the context of medium spiny neurons (MSNs) in the nucleus accumbens, the activation of 5-HT_{1B} receptors on both D1-MSNs (direct pathway) and D2-MSNs (indirect pathway) leads to a decrease in GABA release and possibly the release of neuropeptides as well. Additionally, the dissociated Gβγ subunits can also modulate ion channels, such as inhibiting voltage-gated Ca^{2+} channels or activating certain types of K^{+} channels. These actions contribute to the overall effects of 5-HT_{1B} receptor activation, leading to a decrease in neurotransmitter release.

5-HT_{1B} receptors are widely distributed throughout the brain and are involved in regulating multiple neural circuits and functions. This makes it challenging to determine the specific contributions of these receptors to addiction-related behaviors when using systemic pharmacological manipulations or localized drug infusions that lack cell-type specificity. Studies that use agonists or antagonists to modulate 5-HT_{1B} receptor function may inadvertently overstimulate or block the receptors, which could lead to unintended effects on other brain functions or neural circuits. This issue is particularly pertinent

when the receptors are involved in complex learning processes that require precise temporal and spatial receptor activity control.

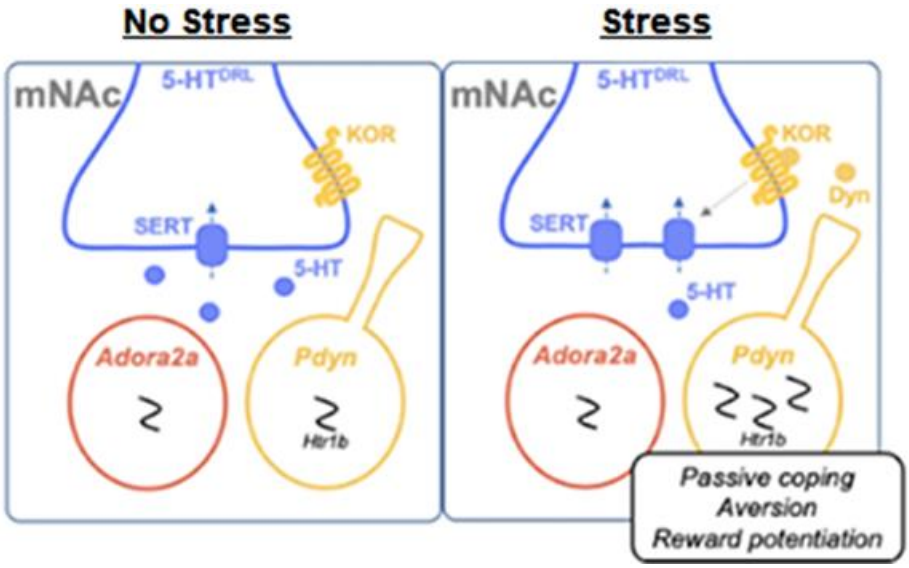


Figure 2.5.III KOR and SERT in the Direct and Indriect Pathways

The role of 5-HT_{1B} receptors in addiction-related behaviors may be influenced by the stage of drug use (acquisition, maintenance, withdrawal) or the specific drug state (e.g., drug-naïve, drug-experienced). These factors can lead to varied and sometimes conflicting findings across studies, as the effects of manipulating 5-HT_{1B} receptors may depend on the specific context in which they are studied. Differences in study design, animal models, drug administration paradigms, and behavioral assays can contribute to discrepancies in the literature regarding the role of 5-HT_{1B} receptors in addiction-related behaviors. Additionally, studies may use different agonists or antagonists with varying selectivity and potency, further complicating the interpretation of results. Overall, the complexity of studying 5-HT_{1B} receptors arises from the diverse roles they play in the brain, as well as the limitations of traditional pharmacological and behavioral approaches.

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CHAPTER 3. THE LATERAL HABENULA AND COCAINE REINFORCED OPERANT RESPONDING

An appropriate amount of rephrasing, reformatting, and reconceptualizing was performed on a previously peer-reviewed publication to allow inclusion in this dissertation. The manuscript was published by Addiction Biology. Sunila Nair was the first author; I co-authored by aiding in editing, experimental design, and execution. The full citation is:

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Section 3.1 INTRODUCTION

3.1.i *Advanced Strategies Using Viral Mediated Gene Transfer*

Viral-mediated gene transfer is a powerful method for delivering genetic material into cell lines or in vivo, allowing researchers to manipulate gene expression and study the function of specific genes or proteins. Adeno-associated viruses (AAVs) are one of the most used viral vectors in neuroscience research due to their low immunogenicity, stable and long-term gene expression, and ability to infect dividing and non-dividing cells. AAVs have a single-stranded DNA genome that is packaged within a protein capsid. There are over 10 serotypes of AAVs, each with a unique capsid structure. The capsid determines the tropism of the virus, or which cell types it can infect, as well as the efficiency of infection in various brain regions. Researchers can choose a specific serotype depending on the cell type and brain region they want to target. One limitation of AAVs is their small packaging capacity, which is around 4.7 kb for most serotypes. This restricts the size of the transgene that can be packaged within the virus. Other Viruses include Herpes Simplex Virus, canine adenosine virus (CAV), and lentiviruses which all have unique patterns of expression in different tissues.

3.1.ii *Chemogenetics: DREADDs, CNO, and Signaling Modulation*

Over the last decade, DREADD technology has been instrumental in advancing our understanding of the neural mechanisms underlying addiction. Several major studies have utilized DREADDs to dissect the roles of specific neuronal populations and circuits in addiction-related behaviors. One such study by Ferguson et al. used DREADDs to investigate the role of nucleus accumbens (NAc) core and shell medium spiny neurons (MSNs) in mediating susceptibility to amphetamine (Ferguson et al., 2009). Other studies have found inhibition of NAc attenuates reinstatement of drug-seeking (McFarland et al., 2004; Nair et al., 2013; Wunsch et al., 2017). Another study by Vardy et al. demonstrated the efficacy of a novel class of DREADDs, called KORDs, that are activated by the synthetic ligand salvinorin B (Vardy et al., 2015). This study expanded the DREADD toolkit for addiction research, allowing for the selective activation or inhibition of distinct neuronal populations within the same animal. In a study focused on the direct and indirect pathways, Creed et al. found that rescuing of the indirect pathway reduced time spent in the reward zone indicating a distinct role for these pathways in mediating cocaine-induced anhedonia (Creed et al., 2016). Chandra et al. used DREADDs to selectively activate or inhibit ventral tegmental area (VTA) dopamine neurons projecting to the NAc or medial prefrontal cortex (mPFC) in rats. The study found that activation of either D1-MSNs or D2-MSNs reduced cocaine-induced locomotor activity, but D1-MSN specifically altered gene expression of their gene of interest similar to cocaine (Chandra et al., 2013). These studies shed light on the importance of further dissecting the neural circuits underlying ventral striatal function by integrating cutting-edge techniques. By selectively manipulating distinct neuronal populations and circuits,

researchers can gain valuable insights into the mechanistic basis of addictive behaviors and identify potential therapeutic targets for addiction treatment.

Developing new techniques and tools, such as chemogenetics and optogenetics, may help to overcome some of these challenges by enabling more targeted and precise manipulation of 5-HT_{1B} receptor function in specific neural circuits and cell types. By utilizing a Cre-dependent system with viral-mediated gene transfer, researchers can achieve selective expression of the hM4Di receptor in specific neuronal populations or pathways of interest. This level of specificity is not achievable with systemic pharmacological manipulations or localized drug infusions. Studying a synthetic receptor designed to target Gi signaling can serve as a good analog for studying 5-HT_{1B} receptors, which also signal through the Gi pathway. There are several advantages to using DREADDs as a chemogenetic tool to investigate the role of specific neuronal populations and signaling pathways in complex brain circuits (Ferguson & Neumaier, 2012, 2015; Vardy et al., 2015). The selective expression of hM4Di receptors in specific neuronal populations enables researchers to investigate how subsets of 5-HT_{1B}-expressing neurons interact within a larger system, such as within the direct and indirect pathways of the nucleus accumbens core. This approach can help elucidate the specific contributions of these pathways to complex brain functions and behaviors. Using DREADDs allows for cell-type specific modulation of Gi signaling, which is not possible with traditional pharmacological manipulations or localized drug infusions. Localized infusions of clozapine-n-oxide (CNO), DREADDs exogenous receptor ligand, is an alternative approach when high or repeated doses of CNO could be problematic (MacLaren et al., 2016; Mahler & Aston-Jones, 2018). This enables researchers to isolate the

effects of Gi signaling on specific neuronal populations and gain insights into their functional roles within a larger neural circuit. DREADDs, such as hM4Di, are activated by CNO. Localized injections have also been used to dissect pathways of collateralizing neuronal projections. For instance, a group differentiated the D1 projections to the SN from the VP during a cocaine-reinstatement test. Their findings highlight the underappreciated role of D1-Projections to the VP as necessary for cue-induced reinstatement of cocaine self-administration, and that D1-projections to the SN do not affect cocaine or cue-induced reinstatement (Pardo-Garcia et al., 2019).

This provides researchers with temporal control over the activation of the synthetic receptor and its downstream effects, allowing for precise manipulation of Gi signaling in specific neuronal populations. This strategy will help understand the role of 5-HT_{1B}-expressing neurons and Gi signaling within the nucleus accumbens core direct and indirect pathways, and potentially uncover novel insights into the complex signaling mechanisms and feedback loops that govern these neural circuits. The complexity of studying 5-HT_{1B} receptors and their role in addiction and other brain functions arises from several factors, including the widespread distribution of these receptors, their involvement in multiple signaling pathways, and their varying roles during different stages of drug use and withdrawal. This complexity may contribute to minor discrepancies in research outcomes.

3.1.iii *The Lateral Habenula in Stress-Related Behaviors*

The lateral habenula (LHb) is a small epithalamic brain structure that has garnered increasing attention in recent years due to its critical role in aversion processing, reward

prediction, and drug abuse. This brain region has emerged as a key player in modulating stress-related behaviors, particularly those associated with cocaine relapse and the incubation of cocaine craving. Research conducted by Sunila Nair and colleagues in the Neumaier Lab at the University of Washington, as well as other research groups, has provided significant insights into the complex interplay between the LHb and various neural circuits and signaling pathways implicated in addiction. The lateral habenula (LHb), an epithalamic nucleus located in the dorsal diencephalon, commonly known to be involved in behavioral flexibility and the processing of aversive information, is an important regulator of midbrain dopaminergic systems (Balcita-Pedicino et al., 2011; Brinschwitz et al., 2010; Geisler & Trimble, 2008; Hikosaka et al., 2008). The LHb receives afferent projections from the limbic forebrain, which is innervated by the cortex, basal ganglia, lateral hypothalamus, and parts of the extended amygdala among other brain regions(Geisler & Trimble, 2008). LHb efferents primarily target brainstem nuclei including the dopaminergic ventral tegmental area (VTA), the GABAergic rostromedial tegmental nucleus (RMTg), the serotonergic dorsal (DRN) and medial raphe nuclei (MRN), and the cholinergic laterodorsal tegmentum among other brain regions (Araki et al., 1988; Herkenham & Nauta, 1979; Jhou et al., 2009). Functionally, LHb lesions increase dopamine (DA) turnover in terminal regions, while local LHb stimulation inhibits spontaneous firing of VTA DA neurons (Christoph et al., 1986; Ji & Shepard, 2007; Lecourtier et al., 2008; Nishikawa et al., 1986). Serotonin (5-HT) neurons in the DRN are also inhibited by LHb stimulation (Park, 1987; Wang & Aghajanian, 1977). Thus, the LHb forms an integrative node between the cortex and brainstem nuclei and is an important regulator of monoaminergic neuronal systems which are known to be involved in

cocaine-taking and -seeking behaviors (Filip et al., 2010). However, little is known about the precise role of this nucleus in operant cocaine self-administration and reinstatement of cocaine seeking. We assessed the effect of transient inhibition of LHb neurons, using adeno-associated viral vectors that express Gi/o-coupled DREADDs (Designer Receptors Exclusively Activated by Designer Drug) (hM4Di) on operant cocaine self-administration and reinstatement of cocaine-seeking induced by a cocaine prime or reexposure to contingent cues. These receptors, created by molecular evolution and site-directed mutagenesis, have lost their affinity for their native ligand, acetylcholine, while gaining high affinity for the synthetic ligand clozapine-N-oxide (CNO) (Armbruster et al., 2007). DREADDs are activated by CNO with nanomolar potency, allowing activation of G-protein coupled signaling depending on which DREADDs are expressed. Following infusion of the viral vectors into the LHb, systemic administration of CNO stimulates hM4Di to activate downstream Gi/o-coupled signaling. We also assessed the specificity of the effects of LHb hM4Di on cocaine self-administration and reinstatement by examining the effect of this manipulation on motor activity, operant food-self administration, and reinstatement of foodseeking behavior.

The LHb receives inputs from multiple brain regions involved in reward and aversion processing, such as the basal ganglia, hippocampus, and amygdala, and sends outputs to midbrain structures like the ventral tegmental area (VTA) and the dorsal raphe nucleus (DRN). This unique position within the brain's neural circuitry enables the LHb to integrate information about reward and aversion and modulate the activity of dopamine and serotonin neurons, thereby influencing emotional and motivational states. A growing body of literature has elucidated the role of the LHb in drug addiction,

particularly in the context of cocaine abuse. Studies have shown that cocaine exposure and withdrawal can alter the activity of LHb neurons, leading to dysregulated signaling within the nucleus accumbens (NAc) and other brain regions involved in reward and motivation. This dysregulation may contribute to the persistence of drug-seeking behavior and the propensity for relapse.

Furthermore, the LHb has been implicated in the incubation of cocaine craving, a phenomenon in which craving for the drug increases over time during abstinence rather than decreasing. Recent research has begun to uncover the molecular and cellular mechanisms underlying this process, with a focus on the signaling pathways that modulate NAc activity. Elucidating the role of the LHb in the incubation of cocaine craving may provide valuable insights into potential therapeutic targets for reducing relapse risk in individuals with cocaine use disorder.

In this chapter, I will provide an overview of the current state of knowledge regarding the role of the LHb in stress-related behaviors, with a particular focus on its involvement in cocaine relapse and the incubation of cocaine craving. We will discuss the seminal work of Sunila Nair and other members of the Neumaier Lab at the University of Washington, as well as research from other groups that have contributed to our understanding of the complex functions of the LHb in addiction. Finally, we will consider the potential implications of these findings for the development of novel therapeutic strategies aimed at targeting the LHb and its associated neural circuits to prevent relapse and improve treatment outcomes for individuals with cocaine use disorder.

Section 3.2 MATERIALS AND METHODS

3.2.i *Cocaine Self-Administration and Reinstatement*

Male Long-Evans rats (Charles River, Raleigh, NC), weighing 325 to 400 g, were used. Rats were double housed initially and allowed to acclimate for at least 1 week to the vivarium prior to the experiment. The temperature- and humidity-controlled vivarium was under a 12-hour light-dark cycle (lights on at 6 AM). Following the acclimation period, rats were injected intracranially with viral vectors and implanted with intravenous catheters into the jugular vein. For cocaine self-administration and reinstatement experiments, food and water were available ad libitum for all rats, except during the 2- to 3-hour training, extinction, and reinstatement session. For food self-administration and reinstatement experiments, rats were on a restricted diet of 18 to 20 g/day (about 70-75% of their regular daily Purina Rat Chow) through the duration of the experiment. The level of food restriction used in our model is on par with the level used in human studies of calorie restriction (Stewart et al., 2013). Further, at this level of food restriction, rats are highly motivated to seek food during reinstatement tests, without excessively extending the duration of extinction training, which is often observed with higher food restriction levels under our experimental conditions ((Nair et al., 2009; Nair et al., 2008). All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were conducted in accordance to the guidelines of the “Principles of Laboratory Animal Care” (Council, 2011).

3.2.ii *Drugs and Virus*

CNO (National Institutes of Health, Bethesda, MD) was dissolved in sterile water with 1 to 2% dimethylsufloxide. The drug was administered by intraperitoneal injection in a volume of 1 mL/kg approximately 20 minutes prior to the test session. Cocaine hydrochloride (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile 0.9% saline and infused in a volume of 0.1 mL at a dose of 0.75 mg/kg/infusion for operant self-administration training. For reinstatement experiments, cocaine (10 mg/kg) was injected intraperitoneally in a volume of 1 mL/kg.

Adeno-associated viral vectors were obtained from the University of North Carolina viral vector core facility (AAV8-hSyn-hM4Di - mCherry [Lot # AV5360d; titer 8.3 X 10 e12] and AAV8-hSyn-hM3Dq-mCherry [Lot # AV5359d; titer 4 X 10 e12]) and Addgene (AAV8-hSyn-eGFP [Titer 3 X 10 e12]). Viral vectors were injected into the LHb stereotaxically (Ferguson et al., 2008; Neumaier et al., 2002). Stereotaxic surgery details are provided in the Supporting Information. Rats, in which at least 90% of cells expressing the reporter gene were confined to the LHb, were included in the analysis.

3.2.iii *Exp. 1-3: hM4Di-Mediated Inhibition of the LHb on Operant Responding*

In Exp. 1, we examined the effect of hM4Di in the LHb on ongoing cocaine self-administration. AAV-hM4Di-hSyn-mCherry was injected into the LHb; rats were implanted with jugular catheters and trained to self-administer cocaine as described. We used a within subjects experimental design with a within-subjects factor of Treatment (Vehicle and CNO 1 or 3 mg/kg), n=6-7 per dose. Thus, each rat was injected

with the vehicle or a single dose of CNO before the test sessions, which were performed in a counterbalanced order. Test days were separated by 1 to 2 regular training days. In Exp 2, Cocaine priming-induced reinstatement Rats were injected with AAV-hM4Di - hSyn-mCherry into the LHb, implanted with jugular catheters, trained to self-administer cocaine (3-h sessions; one session/day), and the operant response was extinguished (n=10). For reinstatement tests, we used a within-subjects experimental design with the within-subjects factors of CNO Treatment (Vehicle or CNO, 3 mg) or Priming Condition (Vehicle or Cocaine, 10 mg). Thus, each rat was injected with Vehicle-Vehicle, CNO-Vehicle, Vehicle-Cocaine, or CNO-Cocaine. The order of injections in the four experimental groups was counterbalanced. Cocaine (10 mg/kg, i.p.; injection volume: 1 mg/mL) or vehicle was injected immediately prior to the test session. In Exp. 3. Cue-induced reinstatement for cue-induced reinstatement tests (n=9), we used a within-subjects experimental design with within-subjects factors of CNO Treatment (Vehicle or CNO, 3 mg) or Priming Condition (No cue or Cue). Thus, each rat was exposed to four experimental conditions: Vehicle-No Cue, CNO-No cue, Vehicle-Cue, and CNO-Cue. The order of vehicle and CNO injections was counterbalanced. The order of cue condition was not counterbalanced in this Exp. to allow for maximum duration between the two cue tests.

3.2.iv *Exp. 4 & 5: Effect of CNO on Reinstatement (No DREADDs)*

The purpose of these was to determine if the activation of Gi/o-coupled signaling in LHb neurons influences locomotor activity. Rats were tested approximately 3 weeks after AAV-hM4Di-hSyn-mCherry was injected into the LHb. Twenty minutes prior to

injection with cocaine (10 mg/kg), rats were injected with either vehicle or CNO (3 mg/kg) (n=8) in a between-subjects experimental design with Treatment (Vehicle and CNO) as the between-subjects factor. Rats were either not injected with viral vectors (Exp. 4) (n=5) or injected with AAV-hSyn-eGFP in the LHb (Exp. 5) (n=4) and trained to self-administer cocaine as described. The effect of CNO (3 mg/kg) or vehicle was tested on cocaine reinforced operant responding in two counterbalanced sessions with at least 24 hours between the two test sessions. The operant response was then extinguished in the absence of contingent cues, and rats were tested for the effect of CNO (3 mg/kg) on cue-induced reinstatement as described in Exp. 3. Subsequently, the operant response was re-extinguished in the presence of cues and the effect of CNO (3 mg/kg) was tested on reinstatement of cocaine seeking induced by a cocaine prime (10 mg/kg) as described in Exp. 2.

3.2.v *Exp. 6: hM3Dq-Mediated Activation of LHb on Operant Responding*

The experimental procedures used were identical to those described in experiments 1 and 3 with the exception that AAV-hM3Dq-hSyn-mCherry was injected into the LHb. The experimental design used to determine the effect of activation of Gq-coupled signaling in the LHb on cocaine- (n=6; Exp. 6A) or food- (n=9; Exp. 6B) reinforced operant responding was a within-subjects factor of Treatment (Vehicle, CNO 1 mg, and CNO 3 mg).

3.2.vi *Statistical Analyses*

Data were analyzed with the statistical program SPSS (GLM procedure). The data on the effect of activation of G-protein coupled signaling in LHb neurons on cocaine and food self-administration were analyzed separately for the number of reinforcers (cocaine infusions or pellets earned) and active lever responding. The data from the reinstatement experiments were analyzed for nonreinforced lever responding on the previously active lever and on the inactive lever. Because the experimental manipulations had no effect on inactive lever responding, which was very low, these data are not reported.

Section 3.3 RESULTS

3.3.i *Exp. 1-3: CNO Inhibition on Operant Responding*

The rats in Expts. 1, 2, 3, 5, and 6 were trained for 10 to 14 sessions and demonstrated reliable cocaine (

***Figure* 3.3**.i, A). Significant increases in active lever responding were observed in food- ($P<.05$) but not in cocaine-trained rats. During the extinction phase, response rates decreased over time in rats previously trained to self-administer cocaine or food pellets (data not shown).

Cocaine self-administration hM4Di -mediated transient inhibition of the LHb significantly increased the number of cocaine infusions self-administered and active lever presses. Two groups of rats (n=6-7/group) were injected with one dose of CNO (1 or 3 mg/kg) or vehicle 20 minutes prior to the test session during which the rats lever pressed for the cocaine. The statistical analyses for each measure (infusions and active presses) included the within-subjects factors of Treatment (Vehicle and CNO 1 or 3 mg) and Time (Session). The analysis for the effect of CNO (1 mg) on the number of infusions earned revealed a significant effect of CNO treatment ($F(1,5) =10.1$, $P=.024$) but no significant effect of Time or Treatment × Time interaction. The analysis for the effect of CNO (1 mg, n=6) on the number of active presses revealed no significant effect of CNO treatment, Time, or Treatment × Time interaction ($P >.05$) (

***Figure* 3.3**.i, B and D). The analysis for the effect of CNO (3 mg) on the number of infusions earned revealed a significant effect of CNO treatment ($F(1,6) = 24.2$, $P=0.003$), Time ($F(3,18) = 12.9$, P.05) (

***Figure* 3.3**.i, C and E).

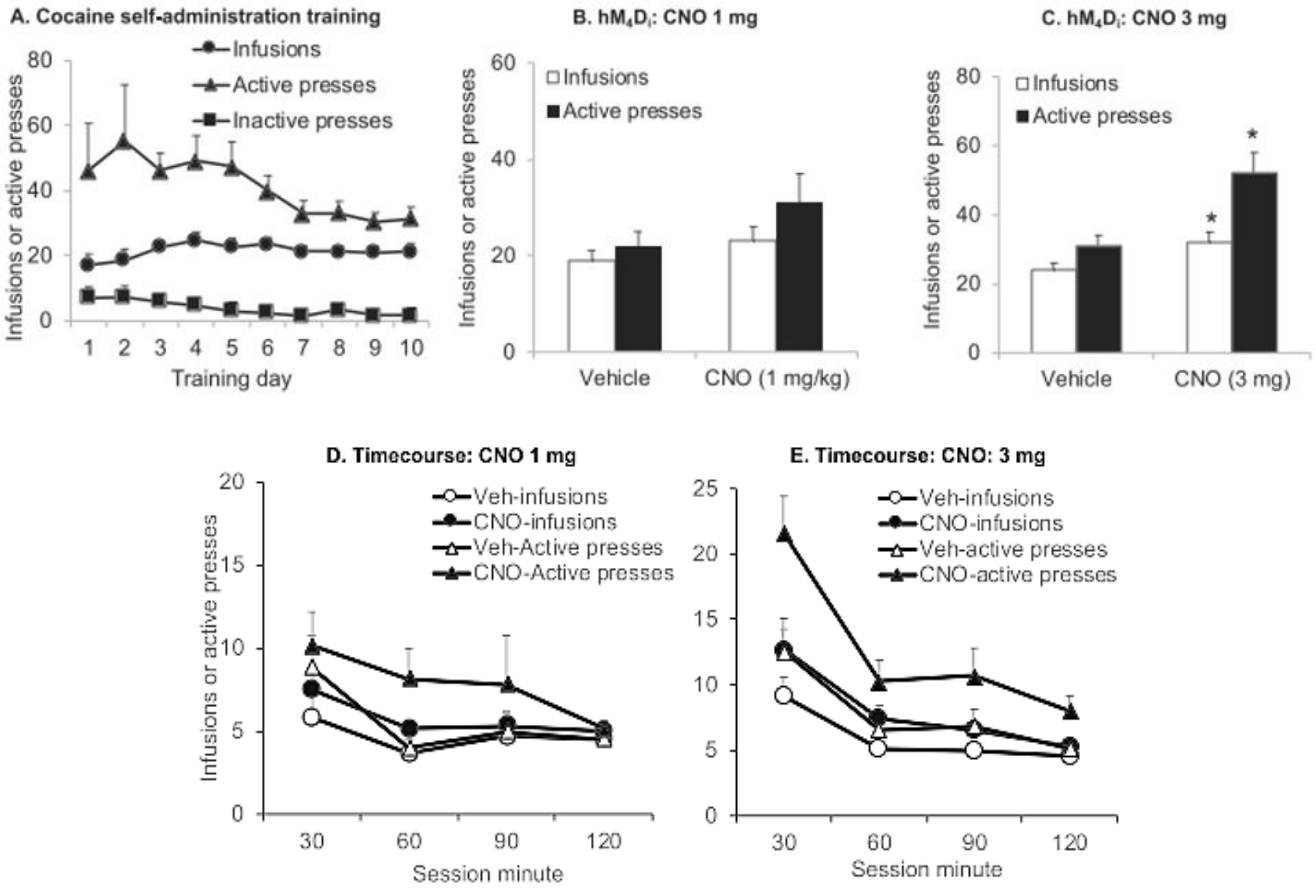

*Figure **3.3**.i hM4Di Inhibition of LHb enhances cocaine self-administration*

3.3.ii *Exp. 1-3: CNO Inhibition on Reinstatement of Cocaine Seeking*

Cocaine priming-induced reinstatement hM4Di -induced inhibition of LHb neurons significantly decreased cocaine priming-induced reinstatement of active lever responding, an effect that was pronounced in the first 30 minutes of the test session (Figure ***3.3.ii***, A and C). The analysis of variance (ANOVA) revealed significant effects of CNO treatment (F(3,27) = 14.8, P.05) (data not shown). Cue-induced reinstatement of cocaine seeking hM4Di -induced inhibition of LHb neurons significantly decreased cue-induced reinstatement of active lever responding (Figure ***3.3.ii***, B and D). The

ANOVA revealed significant effects of CNO treatment ($F(3,24) = 9.2$, P001), Time ($F(5,40) = 34.3$, $P<0.001$), and CNO treatment × Time interaction ($F(15,120) = 6.1$, $P<.001$).

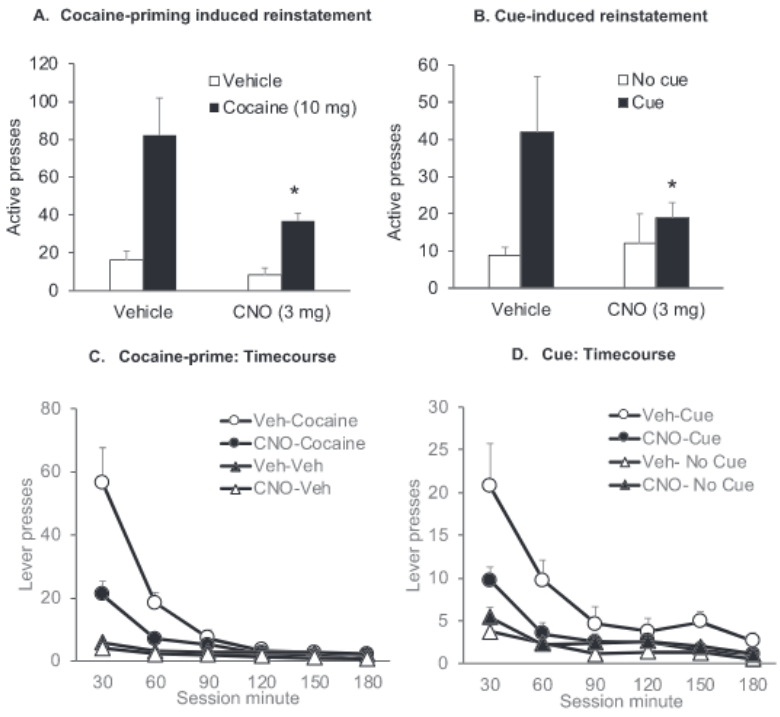

*Figure **3.3**.ii hM4Di Inhibition of LHb reduces cocaine-primed and cue-induced reinstatement*

3.3.iii *Exp. 4-5: No Observable Effect of CNO in Absence of DREADDs*

For operant cocaine self-administration experiments, the statistical analysis included the within-subjects factor of CNO Treatment (Vehicle or CNO). CNO (3mg) had no effect on the number of infusions or active presses in rats where LHb neurons were transduced with AAV-hSyn-eGFP (Figure **3.3.iii**, E-H; n=4) or in rats that did not receive intracranial viral vector injections (Figure **3.3.iii**, A-D; n=5) ($P > 0.05$). For

reinstatement experiments, the statistical analysis included within-subjects factors of CNO Treatment and the Reinstating Stimulus: Priming (Saline or Cocaine) or Cue reinstatement (No cue or cue). Exposure to contingent tone and light cues or a priming injection of cocaine significantly increased active lever responding in both vehicle- and CNO-treated rats. Neither the effect of CNO treatment nor the interaction between CNO Treatment and Priming condition was significantly different ($P<.05$) in rats where LHb neurons were transduced with AAV-hSyn-eGFP (Figure **3.3.iii**, G and H) or in rats that did not receive intracranial viral vector injections (Figure **3.3.iii**, C and D). Together, the data depicted in Figure **3.3.iii** indicate that CNO did not have nonspecific effects on cocaine self-administration or cocaine seeking behavior in the absence of hM4Di expression.

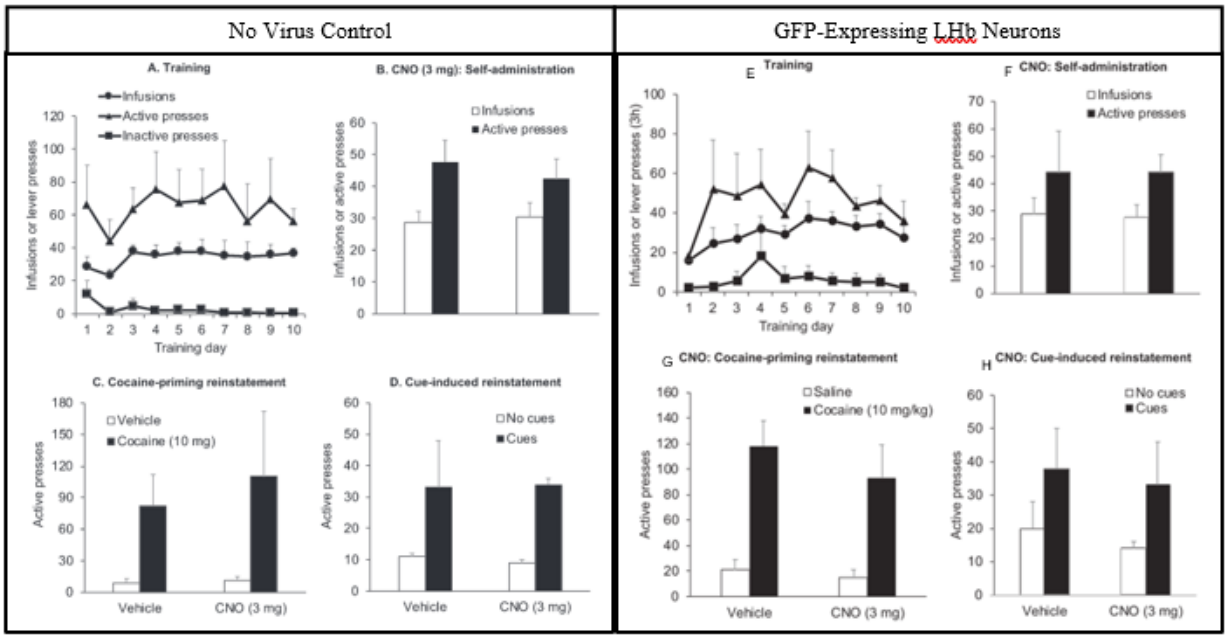

*Figure **3.3**.iii No Effect of CNO on Reinstatement in No-Virus and GFP Control Animals*

3.3.iv *Exp. 6: hM3Dq-Mediated Reduction in Operant Responding*

hM3Dq-mediated transient activation of the LHb significantly decreased the number of infusions and the number of pellets earned (Figure ***3.3.iv***, A and B). The statistical analysis included a within subjects factor of Treatment (Vehicle, CNO 1 mg, and CNO 3 mg) (Infusions: ($F(2,10) = 34.9$, $P<.001$) (n=6).; Pellets: ($F=73.1$, $P<0.001$)(n=9). Cocaine-induced locomotor activity hM3Dq-mediated transient inhibition of the LHb dose-dependently decreased cocaine-induced locomotor activity (Figure ***3.3.iv***, C). The statistical analysis included the effect of Treatment (Vehicle and CNO 0.01, 0.1, 1, and 3 mg) as the within-subjects factor ($F(4,12) = 17.0$, P.05) (n=5). hM3Dq-mediated transient inhibition of the LHb had no effect on motor performance on a rotarod (Figure ***3.3.iv***, D). The statistical analysis included the effect of Treatment (Vehicle and CNO dose) as the within-subjects factor ($P >.05$) (n=5). Effect of hM4Di-mediated inhibition of the LHb on cocaine-induced locomotor activity hM4Di -mediated transient inhibition of the LHb had no effect on cocaine (10 mg)-induced locomotor activity (Figure ***3.3.iv***, E). The statistical analysis included the effect of Treatment (Vehicle and CNO 3 mg) as the between-subjects factor ($P >.05$) (n=8).

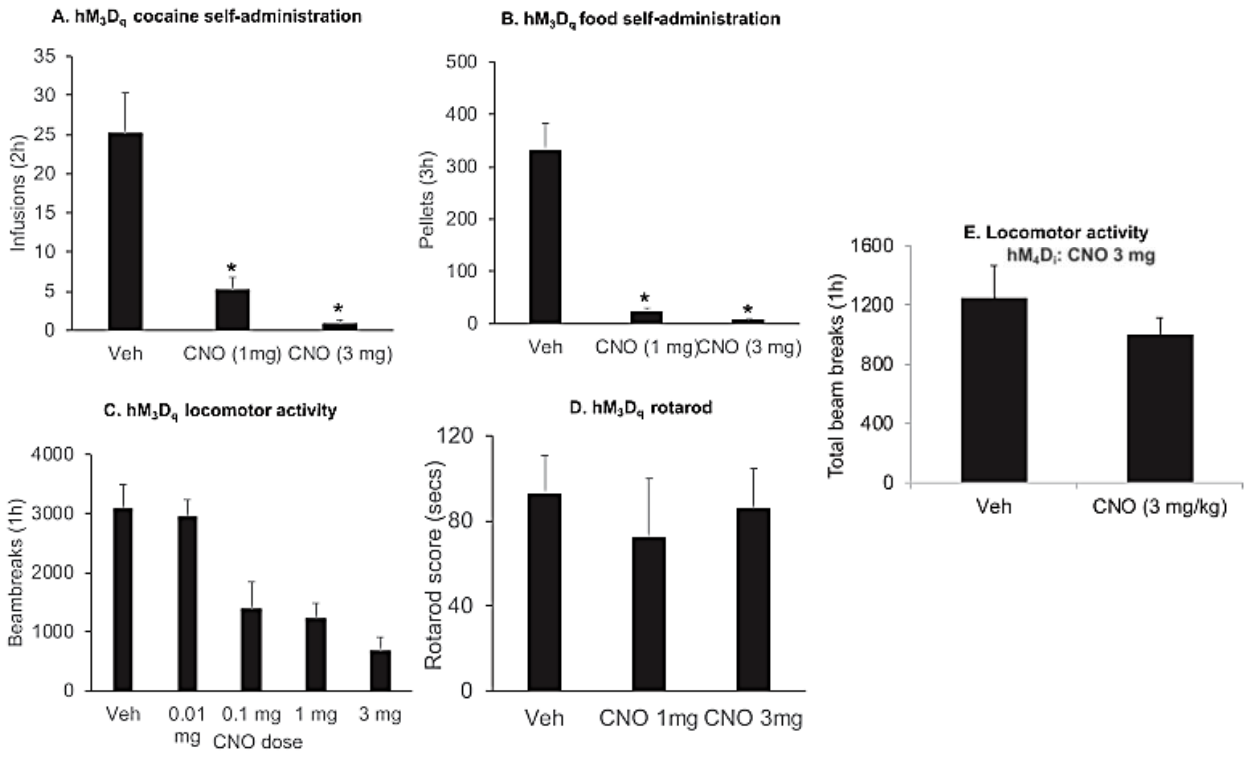

*Figure **3.3**.iv Dose-dependent hM3Dq-mediated reduction in locomotion*

Section 3.4 Discussion

3.4.i *Support for LHb Role in Drug Consumption and Relapse*

Results from our study support two major conclusions. Firstly, chemogenetic inhibition of LHb neurons enhances cocaine but not food self-administration. Secondly, DREADD-mediated transient inhibition of LHb neurons decreases reinstatement of cocaineseeking but not food-seeking. Together, these data suggest that LHb neurons are part of the neuronal circuitries that underlie operant cocaine self-administration and reinstatement of cocaine seeking but not taking and seeking behaviors reinforced by a

nondrug reinforcer. Consistent with these results, CNO-mediated transient inhibition of LHb neuronal activity following hM4Di transduction increased cocaine intake under our experimental conditions. We have recently demonstrated that the 3-mg dose of CNO decreases cocaine (10 mg)-induced c-fos expression in LHb neurons transduced with hM4Di (Coffey et al., 2020). An increase in lever responding for maintenance doses of psychostimulants after an experimental manipulation can be interpreted as being due to a decrease in the rewarding effects of the self-administered drug (Dewit & Wise, 1977; Yokel & Wise, 1976). Our data suggest that transient inhibition of LHb neurons during ongoing cocaine self-administration makes the unit dose of cocaine less rewarding, which results in increased lever responding to maintain levels of cocaine at satiety.

The data also indicate that this effect is pronounced in the drug loading period in the first 30 minutes of the test session. Further studies using more sophisticated behavioral techniques such as the threshold procedure are warranted to fully understand the role of LHb neurons in appetitive and consummatory behaviors reinforced by cocaine (Oleson & Roberts, 2012). A significant advantage of using the chemogenetic approach is that the same manipulation can be used to inhibit as well as stimulate neuronal activity. Under our experimental conditions, CNO-induced transient activation of LHb neurons with hM3Dq (a Gq-coupled DREADD) significantly decreased both operant cocaine and food self-administration. These effects, however, are likely mediated by significant deficits in locomotor activity at the doses of CNO (1 and 3 mg/kg) used to induce activation of Gq-coupled signaling in LHb neurons, despite a lack of effect of these doses of CNO on motor coordination observed on the rotarod. In contrast to cocaine, it has been reported

that there is a lack of effect of electrolytic lesions of the LHb on operant heroin self-administration on both fixed and progressive ratio reinforcement schedules (Wang et al., 2009). This difference is perhaps due to the difference in motivational states produced by cocaine versus heroin (Badiani et al., 2011). The motivational state induced by intravenous heroin self-administration is believed to be entirely appetitive, in contrast to intravenous cocaine that produces a motivational state that is both appetitive and aversive (Ettenberg & Geist, 1993; Ettenberg et al., 1999). Since LHb neurons are known to be activated by aversive stimuli, it is conceivable that LHb neurons are not involved in operant behavior reinforced by heroin (Matsumoto & Hikosaka, 2007). Another potential interpretation of our data is that the effect of hM4Di modulation of LHb activity on cocaine self-administration may be nonspecific, secondary to motor activation. However, the dose of CNO (3 mg/kg) that significantly increased operant cocaine self-administration had no effect on cocaine stimulated locomotor activity, food-reinforced operant responding, or reinstatement of food seeking in animals with hM4Di expression in LHb neurons.

3.4.ii *Controls for CNO side-effects*

In the present study, CNO was used as the exogenous ligand to activate DREADDs. Recent work suggests that CNO may induce nonspecific behavioral effects through conversion of CNO to clozapine (Gomez et al., 2017; MacLaren et al., 2016). To assess the specificity of our experimental findings, we examined the effect of CNO on both cocaine self-administration and reinstatement of cocaine seeking in (a) rats that had no viral vector injections and (b) rats that expressed eGFP in LHb neurons under the control of

the same promoter (human synapsin) and the same virus serotype (AAV8) as rats injected with hM4Di. Our results indicate that CNO injections had no effect on either cocaine self-administration or reinstatement of cocaine seeking. In addition to these direct results, there are four observations, which suggest that 1 and 3 mg/kg injections of CNO do not induce nonspecific behavioral effects under our experimental conditions. Firstly, these doses of CNO had no effect on food self-administration or reinstatement of food-seeking in rats expressing hM4Di in LHb neurons. Secondly, in rats with hM4Di expression in the hippocampus or thalamic nuclei (missed injections), we found no effect of CNO on operant behavior. Thirdly, CNO induced activation of LHb hM4Di had no effect on cocaine-induced locomotor activity. Finally, the back conversion of CNO and subsequent accumulation of clozapine occurs gradually, whereas we observed the largest effects of CNO within the first 30 to 60 minutes of testing.

Our current finding supports a small, but growing body of literature, which suggests that in addition to ongoing operant behavior reinforced by cocaine, neurons in the LHb also mediate reinstatement of drug-seeking behaviors. Studies using c-fos as a marker for neuronal activation demonstrate an increase in density of the immediate early gene in the LHb followed cocaine priming-induced reinstatement of cocaine conditioned place preference, discriminative stimulus, and cue-induced induced reinstatement of cocaine-seeking behaviors (Brown et al., 2010; James et al., 2011; Zhang et al., 2005). Since LHb neurons are activated in response to conditioned cues as well as a cocaine prime, it is not surprising that in the current study, CNO-induced transient inhibition of LHb neurons decreased cocaine priming, as well as cueinduced reinstatement of cocaine-seeking behavior. These results are generally in agreement with pharmacological and

electrochemical studies examining the role of the LHb in the reinstatement of drug-seeking behaviors. Reversibly inactivating the LHb with GABA receptor agonists (baclofen/muscimol) attenuates yohimbine + cue-induced reinstatement of cocaine seeking (Gill et al., 2013). Further, electrolytic lesions of the LHb decrease yohimbine-induced reinstatement of ethanol-seeking behavior (Haack et al., 2014). Meye et al. elegantly demonstrated that disinhibiting neuronal activity in LHb neurons driven by the entopeduncular nucleus decreases forced swim stress-induced reinstatement of cocaine-conditioned place preference (Meye et al., 2016). Our current results taken together with the above-mentioned studies demonstrate a role for LHb neurons in the reinstatement of drug-seeking behaviors, irrespective of the reinstating stimulus. Further, this effect seems to be specific to drugs of abuse since CNO had no effect on the reinstatement of food-seeking behavior under our experimental conditions.

3.4.iii *Future Directions and Importance of Topographical Precision*

Cocaine addiction is a serious, life-threatening disease, treatment options for which are severely limited likely due to our limited understanding of the neuronal circuitry that underlies this disease. Here, we demonstrate a specific role for the LHb in both ongoing short-access cocaine intake (which models recreational cocaine use), as well as reinstatement of cocaine seeking (which models relapse behavior). Our results demonstrate that the LHb is part of neuronal circuitries that underlie both casual drug use as well as addiction. Since we used nonspecific viral vectors in our present study, further studies are warranted to determine the role of specific populations of LHb neurons in modulating these behaviors. In addition, the LHb is broadly divided into

medial and lateral subdivisions, which have vastly different expression profiles of neurotransmitter receptors, neuropeptides, etc (Andres et al., 1999; Geisler & Trimble, 2008). For instance, the DA transporter is primarily expressed in the medial division of the LHb complex along the rostrocaudal length of the brain region. Dissecting the role of the medial and lateral subdivisions of the LHb in cocaine-taking and -seeking behaviors will be a subject for future research.

Section 3.5 References:

Chapter 3

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CHAPTER 4. THE ROLE OF THE VENTRAL STRIATUM IN DRUG DEMAND, REINFORCEMENT, AND THE INCUBATION OF COCAINE CRAVING

4.1 INTRODUCTION

Topographical Differences Along the Dorsal-Ventral and Medial-Lateral Axes

Our understanding of the direct and indirect striatal pathways has evolved over the years, from the discovery of distinct projections to variations in cell-type specific gene expression. The dorsal and ventral striatum are two distinct regions within this system, with differences in receptor expression, projections, and functions. The earliest work on the striatal pathways focused on the identification of distinct anatomical projections. The first model to become widely accepted as the foundation for understanding the basal ganglia circuitry characterized two distinct pathways which bidirectionally modulated behavior in an opposing manner (DeLong, 1990). These two pathways are referred to as the direct pathway (facilitating movement) and the indirect pathway (inhibiting movement). Alexander and Crutcher further expanded this theoretical framework of the basal ganglia, proposing the ability of each individual pathway could, independently, bidirectionally modulate motor control (Alexander & Crutcher, 1990). The discovery of dopamine receptor subtypes (D1 and D2) and their distinct expression patterns in the striatum suggested distinguishable genetic profiles (Lester et al., 1993). Furthermore, Gerfen and colleagues demonstrated that D1 receptors were primarily expressed in the direct pathway, whereas D2 receptors were mainly expressed in the indirect pathway (Gerfen et al., 1990). Research expanded on the differences in gene and neuropeptide profiles between the two: the direct pathway expresses substance P and dynorphin while the indirect pathway expresses neuropeptide Y and enkephalin (Angulo et al., 1995; Brodsky

et al., 2016; Le Moine et al., 1991). Activation by these various neuropeptides results in a wide array of signaling cascades that are thought to be responsible for the nuanced interplay that occurs within and between these heterogenous cell populations (Surmeier, 2007). Gradients of physiological and behavioral impacts are observed along both the dorsal-ventral and medial-lateral axes. Recent research has aimed at understanding the functional implications of the distinct direct and indirect pathways in various aspects of behavior, such as reward processing, habit formation, and decision-making (Kravitz & Kreitzer, 2012; See et al., 2007). The cell bodies of direct pathway and indirect pathway neurons are entirely intermixed, although their axons collateralize local and coalesce into distinct long-range projections. It is important to note that the distinction between direct and indirect pathways are not entirely segregated, and their projections tend to overlap more the further ventral along the axis (Humphries & Prescott, 2010). The dorsal striatum is comprised of the caudate nucleus and putamen; while the dorsolateral striatum is especially involved in motor learning and habit formation, the dorsomedial striatum is important in procedural learning such as action-outcome learning (Eskenazi & Neumaier, 2011; Ferguson et al., 2011; Ferguson et al., 2013). The ventral striatum includes the nucleus accumbens and is involved in reward, motivation, and emotion regulation. While both regions contain direct and indirect pathways, the intermingling of the ventral pathways contributes to the complex functions of the striatum, integrating information from numerous brain regions that are utilized in decision making, action selection, and motor outputs (McFarland et al., 2004; Smith et al., 2013). In summary, the dorsal and ventral striatal pathways differ in their receptor expression, projections, and functions in complex ways that will require precise strategies to differentiate.

Both direct and indirect striatal projections are composed of GABAergic medium-spiny neurons (MSNs); these neurons communicate locally with cholinergic interneurons as well as sending afferent projections to two distinct midbrain regions. In the dorsal striatum, MSNs expressing D1 receptors send terminal projections directly to the output nuclei of the basal ganglia, such as the internal and external globus pallidus, ventral pallidum (VP), ventral tegmental area (VTA) and substantia nigra (SN). More recent interrogations of the direct pathway from ventral striatum discovered that some D1 direct pathway neurons also innervate the VP as well as directly project to VTA and SN. Contrarily, D2 indirect pathway neurons almost exclusively send terminal projections to the VP. Neurons in the VP then indirectly send projections to the subthalamic nucleus (STN), which then projects to the VTA/SNr or reciprocally to the VP, resulting in movement inhibition (Yager et al., 2015). After receiving input from the NAc, the major output regions (SN and VP) project to the thalamus and then back to the cortex which completes the cortico-basal-ganglia-thalamocortical loop (see **Error! Reference source not found.**). The solid lines represent inhibitory projections, dashed lines with arrowhead represent excitatory projections. Green lines are D1-direct projections, red lines are D2-indirect projections, brown lines are thalamocortical projections, and the blue line represents the dopamine input from the VTA/SN.

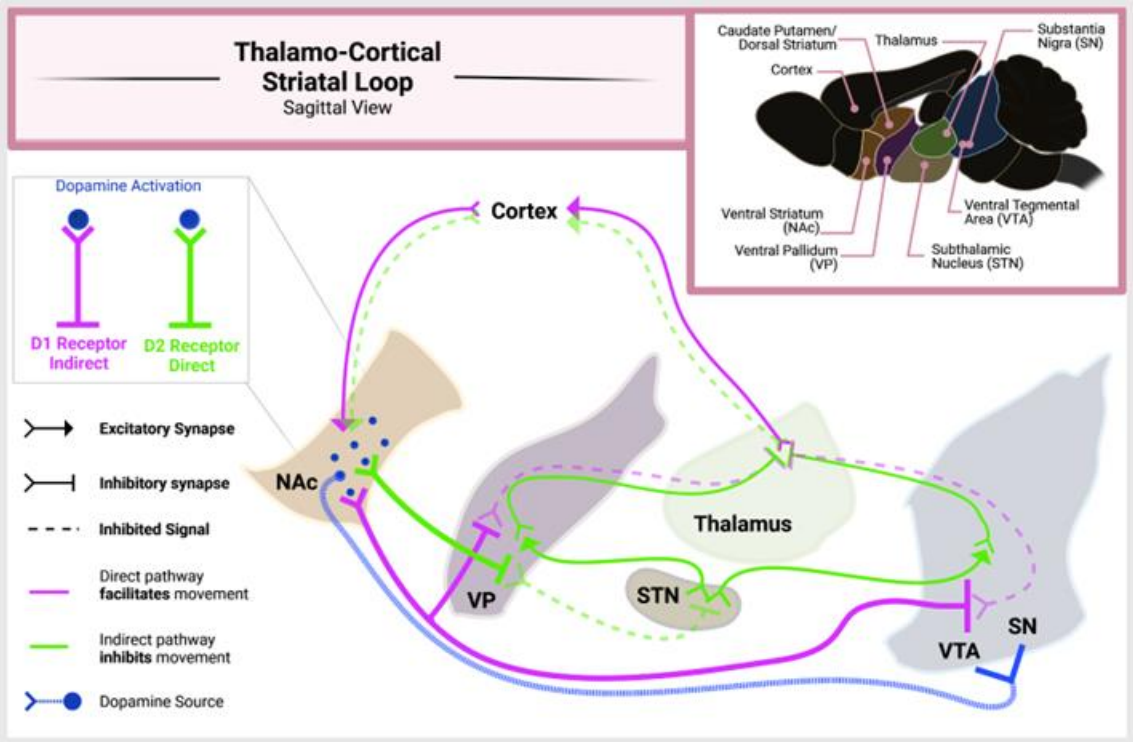


Figure 4.1.i Thalamo-Cortical Striatal Dopamine Loop

MSNs constitute around 95% of striatal neurons, receiving excitatory glutamatergic inputs from the cortex and thalamus, and modulatory dopaminergic inputs from the midbrain (Wunsch et al., 2017). There are also several populations of local interneurons that modulate the activity of the MSNs. For example, GABAergic interneurons tonically inhibit the activity of nearby MSNs—the role of these neurons is likely complex and has not been examined extensively. There are also non-GABAergic interneurons that synthesize and release acetylcholine (ACh); these cholinergic interneurons receive inputs from the cortex, thalamus, and dopaminergic neurons and modulate the activity of MSNs in a complex manner (Berlanga et al., 2003). The ACh-dopamine interaction in the striatum is crucial for motor control, learning, and reward processing (Lee et al., 2020). In the dorsal striatum, cholinergic interneurons can have opposing effects on direct and indirect

pathway MSNs, contributing to the balance between movement facilitation and inhibition. In the ventral striatum, cholinergic interneurons modulate reward-related signaling, affecting the processing of reward prediction errors and the encoding of motivational salience. Kravitz et al., in a separate study, used optogenetics, a method to control neuronal firing with precise spatial and temporal control, finding that the activating the direct pathway induced persistent reinforcement while activating the indirect pathway resulted in transient punishment. They demonstrated these activation patterns resulted in a preference for direct pathway stimulation when compared to indirect pathway stimulation as measured by a modified place preference (Kravitz et al., 2012). Lobo et al. found potentiation of D2 neurons in subsets of rats was inferred from increasing levels of AMPA/NMDA levels correlating with decreasing drug seeking behaviors (Bock et al., 2013). Overall, our understanding of the direct and indirect striatal pathways has evolved significantly over the years, with the contributions of various researchers using various drug-reinforcers. Some misalignments D1 and D2 neurons contribution to cocaine-seeking was minor and excluded because the authors, and I, agreed these differences most likely arose from slight differences in behavioral methodology or analysis technique. Regardless, the midbrain striatal pathways are a crucial component of the basal ganglia circuitry, involved in various motor, cognitive, and reward-related processes. It is important to study the how the dorsal and ventral striatum differ in their output projections, dopamine-expressing populations, and communication involving MSNs and cholinergic interneurons. While the dorsal striatum focuses on motor control and habit formation, the ventral striatum plays a role in reward, motivation, and emotion regulation.

The Notorious Nuances of the Nucleus Accumbens

The nucleus accumbens (NAc) is a key region within the brain's reward circuitry, playing a crucial role in mediating the rewarding and reinforcing properties of natural rewards as well as drugs of abuse. The NAc is located in the ventral striatum, which is part of the basal ganglia, a group of subcortical nuclei involved in various aspects of motor control, learning, and motivation. The NAc is situated ventrally to the caudate-putamen (striatum) and anteriorly to the ventral pallidum, lying at the interface between the limbic and motor systems. The NAc is divided into two major subregions: the core and the shell. The core is more dorsolateral and is functionally and anatomically similar to the dorsal striatum. The shell, on the other hand, is more ventromedial and has connections with limbic structures. These subregions are defined spatially by their location within the NAc, and functionally by their distinct connectivity patterns and roles in reward-related behaviors. From a neuron-type perspective, the NAc comprises these two primary types of medium spiny neurons (MSNs), which make up about 95% of its neuronal population, and interneurons. These MSNs are characterized by their expression of dopamine D_1 or D_2 receptors, defining the direct and indirect pathways, respectively (O'Neal et al., 2020). D_1-MSNs project directly to the output nuclei of the basal ganglia, while D_2-MSNs project indirectly via the external globus pallidus and subthalamic nucleus. This dichotomy is prominent in the dorsal striatum, but there has been observed overlap in the NAc (Humphries & Prescott, 2010; Smith et al., 2013).

The NAc receives afferent inputs from numerous brain regions. The midbrain dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta make important projections to NAc that contribute to reward and learning. Other major inputs

include glutamatergic projections from the prefrontal cortex, hippocampus, amygdala, and thalamus (Baker & Mizumori, 2017; Balcita-Pedicino et al., 2011). In addition, the NAc receives inputs from other neuromodulatory systems like the lateral habenula (LHb) via serotonin, GABA, and dopamine (Coffey et al., 2020). The NAc plays a pivotal role at various stages of the addiction cycle including acquisition, maintenance, and susceptibility to cue-induced relapse following memory extinction. During the acquisition phase, drugs of abuse increase dopamine levels in the NAc, leading to activation of the direct pathway and inhibition of the indirect pathway via D_1 and D_2 receptors, respectively, ultimately promoting drug-seeking and drug-taking behaviors. In the maintenance phase, chronic drug exposure leads to neuroadaptations in the NAc, altering the balance between the direct and indirect pathways and promoting compulsive drug use. During withdrawal and abstinence, the NAc undergoes further neuroadaptations, such as increased glutamatergic transmission and alterations in dopaminergic signaling, which contribute to negative affective states and craving (Koob & Volkow, 2016). These changes increase the susceptibility to relapse, as exposure to drug-associated cues, stress, or drug itself can trigger a reinstatement of drug-seeking behavior. In this context, the NAc serves as a critical hub for integrating multiple inputs and orchestrating complex behavioral responses.

Pathway Specific mRNA Analysis of the Direct and Indirect Pathway

The RiboTag method is a powerful technique that allows for the isolation of ribosome-associated mRNA from specific cell types. This is done by expressing a tagged ribosomal protein in the cell type of interest. The tag can then be used to immunoprecipitate the ribosome and its associated mRNA, allowing for the analysis of the actively translated

mRNA in a cell type-specific manner. We followed the general steps of the RiboTag protocol as described (Gao & Zhao, 2021; Lesiak et al., 2015; Levinstein et al., 2020; Sanz et al., 2019). Understanding the different roles of genes and neuronal networks during active cocaine use, abstinence, and repeated cycles of withdrawal and relapse is crucial because the effects of cocaine on the brain are dynamic and change over time. These changes can contribute to the development of addiction and its persistence even after long periods of abstinence. During active use, cocaine acts on the dopamine system and other neurotransmitter systems, altering neuronal activity, synaptic plasticity, and gene expression. It's essential to study these changes to understand the initial effects of the drug and how they contribute to the development of addiction. During abstinence, the brain undergoes significant changes during abstinence, including alterations in gene expression and neuronal activity, as well as the development of intense cravings for the drug. Studying these changes can shed light on the mechanisms of withdrawal and cravings, which are key factors in relapse. During repeated cycles of use, withdrawal, and relapse, repeated cycles of drug use and withdrawal can lead to neuroadaptations that make the brain more susceptible to addiction and relapse. For example, repeated cycles can lead to an increasingly dysphoric withdrawal state, intensifying cravings and making relapse more likely. Understanding these different stages can help in the development of effective treatments for cocaine addiction. For example, medications could be designed to target specific changes in gene expression or neuronal activity that occur during withdrawal, reducing cravings and the likelihood of relapse.

Intersectional Approach to Pathway Specific Gene Expression

Canine adenovirus (CAV) and adeno-associated virus (AAV) are two commonly used viral vectors in neuroscience research for gene delivery and expression in target cells. CAV is a non-enveloped, double-stranded DNA virus that can infect a broad range of cell types. In neuroscience research, CAV is often used as a retrograde tracer, allowing for the specific targeting of projection neurons by injecting the virus into their downstream target regions (Garcia et al., 2018). CAV can carry large transgene payloads and is capable of long-term gene expression (Junyent & Kremer, 2015). AAV is a small, non-enveloped, single-stranded DNA virus that has low immunogenicity and can infect both dividing and non-dividing cells. AAV vectors are widely used in gene therapy and basic research due to their ability to provide stable and long-term transgene expression with minimal immune response. AAVs have serotypes with varying tropism, allowing for selective targeting of specific cell types or brain regions. The payload capacity of AAV is limited, typically around 4.7 kb, which can be a constraint for larger transgenes. The directional expression of these virus types in conjunction with the conditional expression of Cre-dependent systems enables anatomically specific expression within projection neurons (Nectow & Nestler, 2020). To overcome this limitation of AAV viral capsid capacity and allow for dual expression of two proteins within the same plasmid, researchers have developed strategies such as the use of 2A peptides (Liu et al., 2015). 2A peptides, also known as "self-cleaving" or "ribosome skipping" peptides, are short sequences of amino acids that mediate the co-translational "cleavage" of proteins during their synthesis. When a 2A peptide is inserted between two genes in a single open reading frame, the ribosome will "skip" the peptide during translation, resulting in the production of two separate proteins from the same mRNA molecule. This process is called "ribosome skipping" or "2A-mediated cleavage." Using

2A peptides in AAV vectors allows researchers to express two proteins from a single plasmid, effectively bypassing the packaging size limitation (Lesiak et al., 2015). The 2A peptide sequence can be inserted between the coding sequences of the two proteins of interest, allowing them to be translated as separate polypeptides from a single mRNA molecule. This approach enables the co-expression of multiple proteins in the same cells without the need for multiple promoters or additional regulatory elements. AAV vectors are widely used in neuroscience research for their ability to deliver genetic material into a variety of cell types and brain regions. The use of 2A peptides in AAV vectors allows for the co-expression of two proteins within the same plasmid, overcoming the packaging size limitation of AAVs and enabling researchers to study complex biological processes that require the simultaneous manipulation of multiple genes or proteins.

D_1 and A_{2a} Cre-Expressing Transgenic Rat Strains Offer Genetic Targeting of Ventral Striatal Pathways

Using transgenic CRISPR knock-in cell-inducible rat strains over intersectional viral expression has had several advantages. CRISPR knock-in allows for precise, targeted integration of the desired gene into the host genome at a specific location, reducing the risk of insertional mutagenesis or off-target effects, which can be a problem with viral vector gene delivery. Once integrated, the transgene becomes a part of the host genome and is stably passed on to the progeny of the modified organism. In contrast, viral vector-mediated gene delivery often results in episomal (not integrated into the genome) expression, which can be lost over time or upon cell division. Whole organism modification allows for gene expression changes in every cell of the organism (or in a tissue-specific manner if a specific promoter is used), which may not be feasible with viral

delivery methods due to delivery limitations. Viral vectors can stimulate immune responses, which can limit their effectiveness. This is not a problem with CRISPR/Cas9 gene editing in embryos. The process to generate D_1 and A_{2a} Cre knock-in lines involved several steps. The first step was designing and cloning guide RNAs (gRNAs) targeting the stop codon of the Drd1 and Adora2a genes. Then Cas9 mRNA, gRNAs, and a donor plasmid containing Cre recombinase flanked by homology arms corresponding to sequences upstream and downstream of the stop codon were microinjected into fertilized rat embryos. The injected embryos were transferred into pseudopregnant female rats, and the resulting pups were screened for successful integration of the Cre recombinase at the correct locus. PCR genotyping was used to identify founders (F0 generation) with correct Cre insertion and lines were backcrossed against Long-Evans for five generations. Genome sequencing was used to confirm the precise integration of the Cre recombinase at the correct locus (Pettibone et al., 2019). Immunohistochemistry was used to check for Cre expression in the appropriate brain regions. They examined Cre mRNA expression in different brain regions using quantitative PCR (qPCR) and observed high expression in the striatum, where D1 and A2a neurons are primarily located. They crossed their D1-Cre and A2a-Cre rats with a Cre-dependent reporter line expressing tdTomato and observed cell-specific fluorescence in the striatum, indicating that Cre was selectively expressed in D1 and A2a neurons. They performed immunohistochemistry to assess the co-localization of Cre and D1 or A2a in striatal neurons, which showed high specificity and minimal off-target expression. Initial behavioral assays showed no difference in cocaine-induced locomotion or performance on a Pavlovian conditioned approach when comparing transgenic rats and their littermates.

An alternative approach to cre-inducible gene expression is the generation of bacterial artificial chromosome transgenic technology. Creating these transgenic lines produces reliable exogenous gene expression, without the precision of loci insertion achieved by CRISPR insertion. A different study published in eNeuro used bacterial artificial chromosome-generated D1-Cre and D2-Cre rat lines generated to investigate the role of striatal circuits in reinforcement learning. These transgenic strains were shown to be effective for dissecting cell-type-specific contributions to behavior, as they allowed for the targeted manipulation of D1 and D2 neurons in the striatum during behavioral tasks. The study demonstrated that DREADD-mediated Gi-inhibition of D1 neurons blocked cue induced reinstatement, but interestingly, the result was only seen when stimulating D1 projections to the VP and not in D1 projections to the SN. Furthermore, neither Gi-inhibition of D1 projections to the VP and SN prevented cocaine-induced reinstatement (Pardo-Garcia et al., 2019). This indicates a specific role for the D1 striatopallidal projections that is functionally distinct from striatonigral projections or collaterals. This finding further supports the integrative and necessary role of the ventral pallidum in reinstatement. It is possible that the ability of the VP to attenuate drug craving is impaired during consumption.

Behavioral Economics, Demand Curves, and Elasticity

In recent years, behavioral economics models have emerged as a powerful tool for studying rodent models of cocaine abuse. These models, drawing upon the work of researchers such as Gary Aston-Jones and Alex Kawa enable the investigation of the relationship between drug demand and price, as well as the factors that influence these relationships (Bentzley et al., 2013; Kawa et al., 2016). A key component of behavioral

economics models is the generation of fitted demand curves, which are used to study the elasticity of demand for cocaine. Elasticity refers to the sensitivity of consumption to changes in price, with more elastic demand being associated with a greater reduction in consumption in response to price increases (Hursh & Silberberg, 2008). Fitted demand curves can be obtained by varying the cost of cocaine (e.g., the number of lever presses required) and measuring the resulting changes in consumption. Two important parameters derived from demand curves are Q0 (consumption estimated at zero cost) and Pmax (price at which maximal expenditure occurs). Q0 values provide insight into the baseline consumption of cocaine, while Pmax values indicate the price at which demand is most sensitive to changes (Oleson & Roberts, 2019). These parameters can be instrumental in understanding the factors that drive drug-seeking behavior and in developing targeted interventions. The use of α (alpha) as a metric to evaluate the reduction in cocaine value at rising costs is particularly beneficial, as it allows for the quantification of the rate at which demand decreases with increasing price. Higher α values indicate a more rapid decline in demand as the price increases, reflecting a lower valuation of cocaine (Bentzley et al., 2014).

Behavioral economics models differ from other reinforcement schedules, such as progressive ratio and variable ratio, in several keyways. While progressive ratio schedules involve increasing the response requirement with each subsequent reinforcement (i.e., progressively increasing the "cost" of the drug), and variable ratio schedules involve providing reinforcement after an unpredictable number of responses, behavioral economics models examine the interplay between consumption and price systematically across a range of prices (Bickel et al., 2014). This approach allows researchers to glean

valuable insights into the factors influencing drug demand and addiction vulnerability, thereby informing the development of improved treatment strategies.

The quarter logarithmic decrease, employed in the experimental design of some behavioral economics studies, involves systematically reducing the value of reinforcers available in a session by one-quarter logarithmic units, thereby allowing for the assessment of demand across a wide range of reinforcer amounts (Hursh & Winger, 1995). This method entails manipulating the price of the drug by adjusting the response requirement (e.g., the number of lever presses needed to obtain a single drug infusion) and measuring consumption at each price level. The resulting data points are fit to an exponential demand equation, which captures the relationship between price and consumption, allowing for the determination of key demand parameters, such as Q0, Pmax, and α. One major advantage of this methodology is its ability to systematically evaluate the reinforcing value of drugs across a range of prices, thereby providing a comprehensive understanding of the factors that influence drug-seeking behavior. This approach also allows for the direct comparison of the reinforcing properties of different drugs, as well as the examination of individual differences in demand parameters, which can inform the development of targeted interventions for substance use disorders (Bentzley et al., 2013). However, there are some limitations to this methodology. The quarter logarithmic decrease method may be time-consuming and labor-intensive, requiring multiple experimental sessions to obtain a full demand curve. Additionally, this method may not be suitable for all drug classes or administration routes, as some drugs may have ceiling effects or other pharmacokinetic limitations that could affect the interpretation of demand curves (Bentzley et al., 2014).

Despite these limitations, the application of the quarter logarithmic decrease method in behavioral economics studies has significantly advanced our understanding of the reinforcing values across multiple drugs of abuse. By providing a systematic and quantitative approach to assess drug demand, this methodology has allowed researchers to directly compare the reinforcing properties of different substances, identify factors that influence vulnerability to addiction, and inform the development of more effective treatment strategies for substance use disorders. The application of behavioral economics models to the study of rodent models of cocaine abuse has yielded significant insights into the relationship between drug demand, price, and addiction-related behaviors. These models, by using fitted demand curves and parameters such as Q0, Pmax, and α, have provided a more nuanced understanding of the factors driving drug-seeking behavior further expanding upon available techniques. The goal of the following set of experiments was to explore differences in the translatome and behavior following manipulations of the direct and indirect pathway during drug maintenance and prolonged withdrawal.

4.2 Experimental Designs

Relapse is a complex symptom that can be triggered by stress, drug exposure, or drug-related cues. The goal of Experiment 1 is to determine the effect of inhibiting striatopallidal MSNs on demand curves generated using within-session threshold procedures. This advances the field because to the best of my knowledge, DREADD manipulation during behavioral economics training has not been assessed in the ventral striatum. These findings are especially important in dissecting the role of ventral striatal pathway activity on drug craving, drug seeking, and determining value as a function of price. Understanding how cocaine impacts the functioning of the limbic system is

clinically relevant because of the prevalence of drug-induced relapse. The goal of Experiment 2 is to determine the effect of inhibiting direct or indirect pathways during the incubation of craving relapse test. These studies differ in their approach to targeting the direct and indirect pathway (intersectional viral or transgenic strains) and the stage of addiction being assessed. In experiment 1, brains were processed for target validation using immunohistochemistry. In experiment 2, brains were processed for RiboTag mRNA analysis. Table 4.4.i summarizes the rat strains, viruses, and sample sizes used in each experiment.

4.3 Experiment 1: Within-Session Threshold Materials and Methods

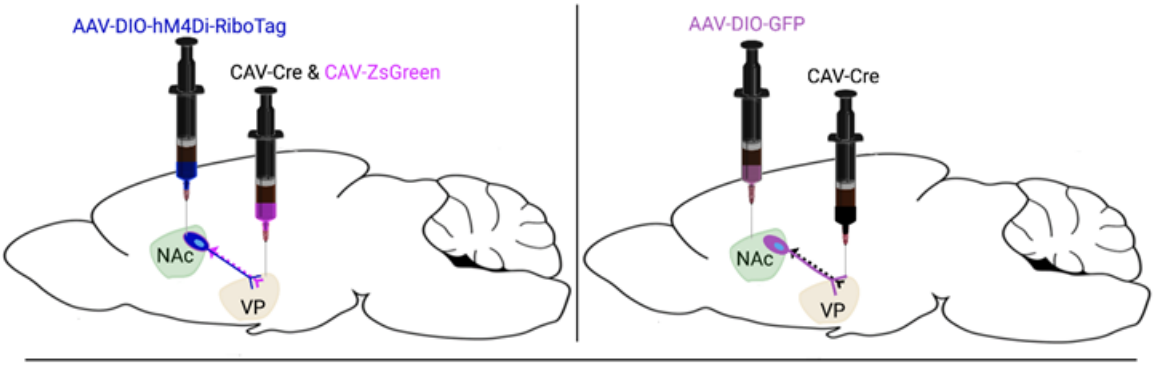
Exp. 1: Animals and Husbandry

Male Long-Evans rats (Charles River, Raleigh, NC), weighing 325 to 400 g, were used in the behavioral economics and punishment experiments. Rats were double housed throughout the experiment except in situations of their cage mate's death. The temperature- and humidity-controlled vivarium was under a 14-hour light- 8-hour dark cycle (lights on at 6 AM). After acclimating for at least 1 week to the vivarium prior to the experiment, rats underwent surgeries.

Exp. 1: Stereotaxic Injections and Catheter Implant Surgeries

The ventral striatal pathways were targeted by dual infection of intersecting viruses. To target the striatopallidal, or indirect, pathway, CAV-Cre (with 1% CAV-Zs-Green) was injected bilaterally into the VP and AAV-DIO-hM4Di-RiboTag was injected into the NAc core. As a control, CAV-Cre without Zs-Green was injected into the VP and AAV-DIO-

eGFP was injected into the NAc core (Paxinos & Watson, 2013). In the rats injected with AAV-DIO-GFP, Zs-Green was not mixed with CAV-Cre to ensure NAc viral expression



Intersectional Viral Targeting of Striatopallidal Projections

resulted from the AAV. The coordinates for each region were as follows: NAc (AP: +1.9, ML: ±1.5, DV: -8.1) and VP (AP: 0.0, ML: ±2.0, DV: -8.7). The AAV-DIO-viruses only express in neurons where the Cre-recombinase was retrogradely expressed. **Error! Reference source not found.** diagrams the retrograde Cre and Zs-Green expresssion (which we attempted to use for targeting validation) and the local expression of RiboTag speifically in MSNs projecting to the Cre+ neuronsBecause of the time required to complete four injections, the viral surgeries were completed in one session and rats were allowed to recover for 7 days before undergoing a vascular surgery where a catheter was implanted into the rat's right jugular vein. Rats were returned to their cages for 5 more days of post-operative monitoring prior to self-administration training.

Figure **4.3**.ii Intersectional Viral Infusions for Targeting Anatomical Projections

Exp. 1: Drugs and Treatment

Clozapine-N-oxide (CNO, National Institutes of Health, Bethesda, MD) was dissolved in sterile water with 1 to 2% dimethylsulfoxide. The drug was administered at a dose of 3

mg/kg by intraperitoneal injection in a volume of 1 mL/kg approximately 20 minutes prior to the test session. Cocaine hydrochloride (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile 0.9% saline and infused in a volume of 0.1 mL at a dose of 0.75 mg/kg/infusion for operant self-administration training. For within-session threshold, the concentration of cocaine in solution was 5 mg/mL.

Exp. 1: Behavioral Economics Training Timeline

Rats were trained to self-administer (SA) cocaine over ten daily sessions. Each training session lasted 3.5 hours on an FR-1 reinforcement schedule with a 20-second timeout period, 6-second light cue, and 0.75mg/kg cocaine infusions delivered in 0.1mL over 3.5 seconds. A 15-minute period without drug availability separated each hour of drug access. The criterion for stable responding during SA training was a minimum of 25 infusions during a 3-hour session with pattern-like responses each hour. Each hour was separated by 3 minutes. In a separate group, 5 Long-Evans male rats were trained over 10 daily 6-hour sessions under the same operant conditions. Each hour of the session was separated by a 3-minute break. Once stable responding was sustained for a minimum of 5 days before, rats began the 110-minute within-session threshold procedure. In these two-hour sessions, the cocaine dose was decreased on a quarter logarithmic scale by reducing the duration of the pump infusion every 10 minutes (422–1.3 μg/infusion). There was no timeout period between infusions; active lever presses during infusions were counted but did not precipitate an infusion. Rats were trained on this 110-minute threshold procedure for a minimum of 5 days prior to CNO testing. Approximately 20 minutes before the threshold test, rats were injected with either 3mg/kg CNO or vehicle (1mL/kg). Twenty-

four hours later, rats were injected with the counterbalanced treatment and then tested in the same manner.

Exp. 1: Transcardial Perfusions

Rats were euthanized with an intraperitoneal injection of pentobarbital sodium. The procedure proceeded once the rat was unresponsive to paw pinch and upon absence of corneal reflex. Perfusions were performed with 100 mL phosphate-buffered saline (PBS) solution followed by 200 mL of 4% paraformaldehyde, both at pH 7.4 and kept on ice. Brains were removed and postfixed in 4% paraformaldehyde overnight, then placed into PBS. To cryoprotect the brains prior to sectioning, brains were transferred to a 30% sucrose solution until they no longer floated (after about 2-4 days). Brains were sectioned on a cryostat at 40 microns and stored in 1x PBS in 24 well plates.

Exp. 1: Free-Floating Perfused Tissue Immunohistochemistry

Sections were transferred to a 12-well plate using a paintbrush. Wells were blocked in 10% normal goat serum (NGS) with 0.1% Triton X-100 in PBS for a duration of 60 minutes. Sectioned were moved to a well containing Rabbit anti-HA monoclonal antibodies (Cell Signaling, HA-Tag-C29F4, #3724) was diluted 1:1000 in 1% NGS and incubated at 4°C overnight. The sections were rinsed in 1x PBT twice, each for 5 minutes, and then transferred into wells with 1:1000 of the secondary antibody in PBS with 0.01% Triton X-100, goat-anti-rabbit Alexa Fluor™ 488 (Thermo Fisher, # A27034) for 1 hour. Sections were kept in the dark during the incubation steps. Afterwards, the sections were sequentially rinsed twice in PBS with 0.1% Triton X-100 for 2 minutes, and finally in plain 1x PBS for 2 minutes. Sections were put into a dish of H2O with the depth of the water

allowing for swift maneuvering and mounting of sections to slides with a fine paintbrush. After allowing the slides to dry marginally to eliminate excess liquid, they were cover-slipped with prolong gold and DAPI and stored in a dark place for at least 24 hours before imaging.

4.4 Experiment 2: Incubation of Craving Materials and Methods

Exp. 2: Animals and Colony Maintenance

The temperature- and humidity-controlled vivarium was under a 14-hour light- 8-hour dark cycle (lights on at 6 AM). For the incubation of craving experiment, breeder colonies were setup for D1-cre expressing and A2a-cre expressing Long-Evans transgenic rats ordered from Rat Resource and Research Center (LE-Drd1em1(iCre)Berke RRRC#: 856; LE-Adora2aem1(iCre)Berke RRRC#: 857). Breeders were set up with Long-Evans rats (Charles River, Raleigh, NC) to allow for timed litters; the sex of the heterozygote parent was alternated between generations. Pups were genotyped at 3 weeks old according to protocols provided by strain creators. Wild-type pups were culled and confirmed heterozygotes (A2a-Cre or D1-Cre) were weaned and males remained double housed throughout the experiment while females were triple housed (except in situations of their cage mate's death). Surgeries were performed on rats 8-12 weeks old.

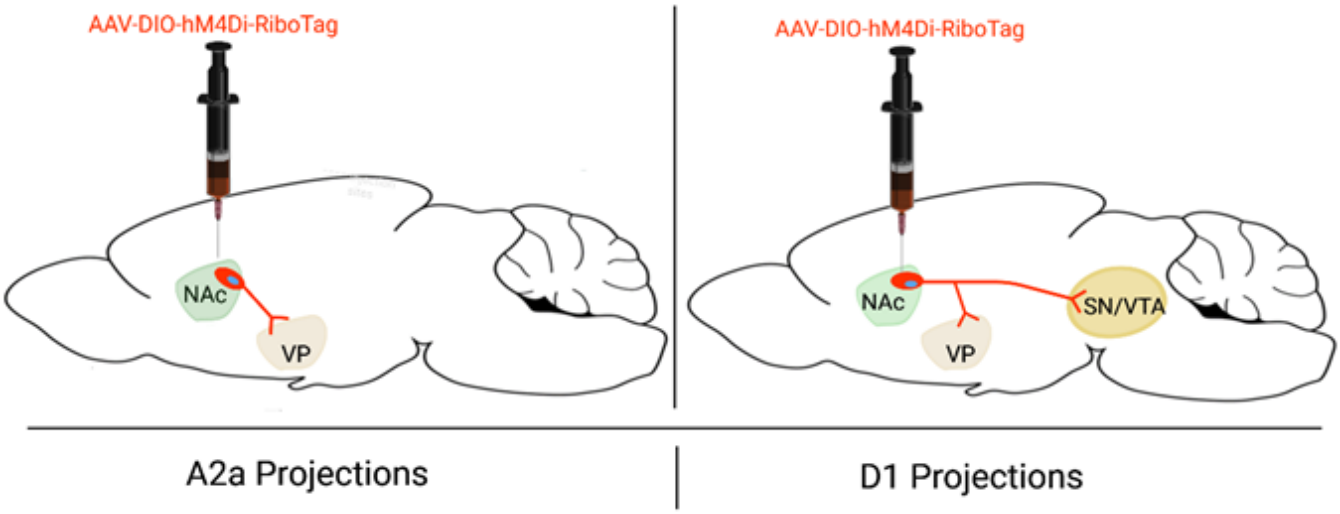
Exp. 2: Drugs and Treatment

For this experiment, the intracranial viral surgeries described below were coordinated with catheter implantation to allow both procedures to be completed in one surgical session. Rats were given 5-8 days of post-operative monitoring before beginning behavioral training. For the first three days of self-administration, rats were on a

restricted diet of 18 to 20 g/day (about 70-75% of their regular daily Purina Rat Chow). After the third day of training, food and water were available ad libitum for all rats for the remainder of the experiment. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were conducted in accordance to the guidelines of the "Principles of Laboratory Animal Care" (Council, 2011). CNO (National Institutes of Health, Bethesda, MD) was dissolved in sterile water with 1 to 2% dimethylsulfoxide. The drug was administered at a dose of 3 mg/kg by intraperitoneal injection in a volume of 1 mL/kg approximately 20 minutes prior to the test session. Cocaine hydrochloride (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile 0.9% saline and infused in a volume of 0.1 mL at a dose of 0.40 mg/kg/infusion for operant self-administration training. It's important to note that the dose of cocaine was reduced from 0.75mg/kg/infusion throughout the entirety of the self-administration training.

Exp. 2: Stereotaxic Injections and Catheter Implants in Transgenic Rats

The ventral striatal pathways were targeted by bilateral infection of AAV-DIO-hM4Di-RiboTag directly into the NAc core of D1- or A2a- expressing neurons to target the direct



and indirect pathways, respectively (AP: +1.9, ML: ±1.5, DV: -8.1). The Cre-inducible virus was expressed in the direct or indirect pathway as shown in .

Figure **4.4**.iii Viral Infusions for Targeting Genetically Distinct Pathways

Exp. 2: Incubation of Craving Behavioral Training Timeline

Rats were trained to self-administer (SA) cocaine over ten daily sessions. Each training session lasted 3.5 hours on an FR-1 reinforcement schedule, 4-second light cue, and 0.40mg/kg cocaine infusions delivered over 2-4 seconds (adjusted according to weight). The 4-second cue served as the sole timeout period between lever presses. The first 6 days of training consisted of three bouts of 1-hour drug access separated by 15 minutes of no drug availability. On SA days 7-10, the drug availability shifted to five bouts of 30-minute drug-access followed by 15-minutes of no drug availability. Criterion for stable responding during SA training will be a minimum of 25 infusions during a 3-hour session with pattern-like responses each hour. Upon completion of the 10 training days, rats were returned to their home cage. The following probe tests were conducted in the same manner as the training, except the tests only lasted 30 minutes and active lever presses did not result in infusions of cocaine (visual and audio cues were still presented). The first withdrawal probe test occurred on the second day of abstinence. Rats were handled 2-3 times per week during their forced abstinence. The final withdrawal day probe test occurred on day 35 of abstinence. For this experiment, animals were only included in the analysis if they completed Day 2 and Day 35 withdrawal tests. 56 rats were included: 13 A2a-Cre females, 14 A2a-Cre males, 11 D1-Cre females, and 18 D1-Cre males. Each of these

four genotype-sex groups was further split between a CNO-Treated and Vehicle treated group for the Day 35 incubation test.

Exp. 2: RiboTag Fresh Brain Extractions

Brains were rapidly extracted and rinsed with ice cold homogenization buffer. The ventral striatum was then microdissected using surgical razor blades atop a foil covered ice block. The microdissection consisted of coronal-oriented trimming of the prefrontal cortex and everything posterior to an estimated bregma. The lateral cortices were then trimmed. The corpus collosum and dorsal striatum were trimmed half -way down the ventricles and most ventral portion below the anterior commissures were trimmed when possible. The remaining "punch" was then homogenized using a fitted drill press in 2mL supplemented homogenization buffer (HB: 500µL per well, 50mM Tris-HCl, 100mM KCl, 12mM MgCl2, 1% NP-40, 1mM DTT, 1× Protease Inhibitor Cocktail (Sigma), RNasin 200 Units/mL (Promega, Madison, WI), cyclohexamide 100ug/mL (Sigma), Heparin 1mg/mL (APP pharmaceuticals, Lake Zurich, IL)). Samples were centrifuged at 4°C at 11.9 × g for 10min, and supernatant was collected. 100µL of the supernatant (5%) was saved as the input fraction and immediately stored at −80°C. The remainder of the supernatant became the RiboTag-IP samples and were processed for immunoprecipitation. 5µL of mouse monoclonal HA-specific antibody (HA.11, ascites fluid; Covance, Princeton, NJ) was added and samples were rotated at 4°C for 4 hours to allow indirect antibody binding. 300µL of protein A/G magnetic beads (Promega) were washed with HB prior to addition to RiboTag-IP fractions and rotated at 4°C overnight. The next day, RiboTag-IP fractions were placed on DynaMag-2 magnet (Life Technologies) on ice, and the bead pellet had three 10-minute washes with high salt buffer (HSB: 50mM Tris, 300mM KCl, 12mM

MgCl2, 1% NP-40, 1mM DTT, and 100ug/mL cyclohexamide). After the final wash, HSB was removed and beads were re-suspended in 400µL of supplemented RLT plus buffer (10µL betamercaptoethanol/10mL of RLT Buffer) from the RNeasy plus micro kit (Qiagen, Hilden, Germany) and vortexed vigorously. RLT buffer containing the immunoprecipitated RNA was removed from magnetic beads prior to RNA purification using RNeasy Kit according to manufacturer's protocol. Likewise, 350µL of RLT was added to Input fractions prior to RNA isolation using RNeasy Kit; input fractions were processed with RNAeasy plus mini kit (Qiagen, Hilden, Germany). RiboTag-IP samples were eluted in 16µL of water, and RiboTag-Input samples were eluted into 40uL of water.

Exp. 2: First Strand cDNA Synthesis and qPCR

RNA concentration was measured using Nanodrop and Quant-iT RiboGreen (Life Technologies) methods. 15–30ng of RNA was reverse transcribed using SuperScript VILO Master Mix (Life Technologies). A cDNA standard curve was generated from surplus input fraction RNA then serially diluted to run a standard curve for each gene target. After cDNA synthesis, cDNA was diluted to final working concentration of 0.1ng/µL "relative RNA" (quantity of RNA added to cDNA synthesis reaction). qPCR experiments were carried out on Viia7 Real Time PCR system (Life Technologies) using SYBR Select Master Mix (Life Technologies). Cycling conditions: 50°C 2 min, 95°C 2 min, 40 cycles of 95°C 15 s and 62°C 45 s, followed by a melt curve. For primer list see

HTR1Brat FWD Set 1	CACTGATGCGGTGGACTATT

HTR1Brat REV Set 1	AGCGAAATCGAGATGGAGAAG
HTR1Brat FWD Set 5	CGACGACTACATTTACCAGGAC
HTR1Brat REV Set 5	CCGATACACCGTAGCGATTAC

Table 4.6.ii. A standard curve was prepared by serial dilution of pooled RNA samples. Samples were analyzed in triplicate. Relative starting quantity was calculated based on CT-values and interpolation using standard curve samples for each series of experiments. For all analyses except for direct measurement of housekeeping genes (GAPDH and ß-Actin), expression was normalized using the housekeeping gene GAPDH. Housekeeping gene quantity was consistent across samples within RiboTag-IP and Input fractions, but RiboTag-IP RNA levels were consistently lower than Input. Therefore, normalization factors were generated based on average housekeeping levels for RiboTag-IP fractions and Input fractions independently.

Table 4.4.i Sample Size Per Treatment Group

Experiment	Strain	Virus	Sex	Treatment	n
Exp 1: Within-Session Threshold	Long-Evans	DIO-hM4Di-RiboTag	Male	CNO & Veh	7
Exp 1: Within-Session Threshold	Long-Evans	DIO-GFP	Male	CNO & Veh	5
Exp 2: Incubation of Craving	Drd1-Cre	DIO-hM4Di-RiboTag	Female	Veh	6
Exp 2: Incubation of Craving	Drd1-Cre	DIO-hM4Di-RiboTag	Female	CNO	5
Exp 2: Incubation of Craving	Drd1-Cre	DIO-hM4Di-RiboTag	Male	Veh	10
Exp 2: Incubation of Craving	Drd1-Cre	DIO-hM4Di-RiboTag	Male	CNO	8
Exp 2: Incubation of Craving	A2a-Cre	DIO-hM4Di-RiboTag	Female	Veh	7
Exp 2: Incubation of Craving	A2a-Cre	DIO-hM4Di-RiboTag	Female	CNO	6
Exp 2: Incubation of Craving	A2a-Cre	DIO-hM4Di-RiboTag	Male	Veh	9
Exp 2: Incubation of Craving	A2a-Cre	DIO-hM4Di-RiboTag	Male	CNO	5

Exp. 2: On-Slide Fresh-Tissue Immunohistochemistry

Some brains were processed for RiboTag which prohibited validation with immunohistochemistry. Brains were equilibrated and sectioned in a cryostat at 16microns. After mounting, slides were post-fixed a 15-minute treatment in 4% paraformaldehyde (PFA) at 37°C, followed by ethanol dehydration, first at 70% for 5 minutes and then with fresh 100% ethanol for 10 minutes. After brief air-drying of 2-5minutes, hydrophobic barriers were drawn tightly around the sections. Slides were then blocked in a 10% normal goat serum (NGS) solution with 0.1% Triton X-100 in PBS for a duration of 60 minutes. The hydrophobic barrier allowed 100-300uL of antibodies to be used per slide. Rabbit anti-HA monoclonal antibodies (Cell Signaling, HA-Tag-C29F4, #3724) was diluted 1:250 in 1% NGS and incubated at 4°C and 23°C overnight or for 4

hours, respectively. Following incubation, the sections were rinsed in 1x PBT twice, each for 5 minutes. The secondary antibody, goat-anti-rabbit Alexa Fluor™ 488 (Thermo Fisher, # A27034) was then applied at 1:1000 for 1 hour at room temperature. During this incubation period, the samples were kept in the dark. The dilution of secondary antibodies is done in PBS with 0.01% Triton X-100. Afterwards, the sections were sequentially rinsed twice in PBS with 0.1% Triton X-100 for 2 minutes, and finally in plain 1x PBS for 2 minutes. DAPI was applied for 30 seconds before cover-slipping.

4.5 EXPERIMENT 1: WITHIN-SESSION THRESHOLD RESULTS

Exp. 1: Zs-Green Retrograde Labeling of NAc Neurons

Intersectional surgeries required the precision of four accurate infusions targeting the dorsomedial VP projections to the NAc core. Expression was confirmed via immunohistochemistry. Zs-Green was able to be imaged in perfused and sectioned brain tissue without antibody staining. Some brains were processed for RiboTag which prohibited validation with immunohistochemistry. Strong retrograde labeling of cell bodies from projections to the VP are seen in the NAc core and shell. In the left panel of Figure 4.5.iv, NAc cell bodies are indicated inside the yellow outline. Localized AAV-expression within the NAc core allowed for specific targeting despite potential range of CAV-expression because AAV-expression is much more localized than Zs-Green (Figure 4.5.iv).

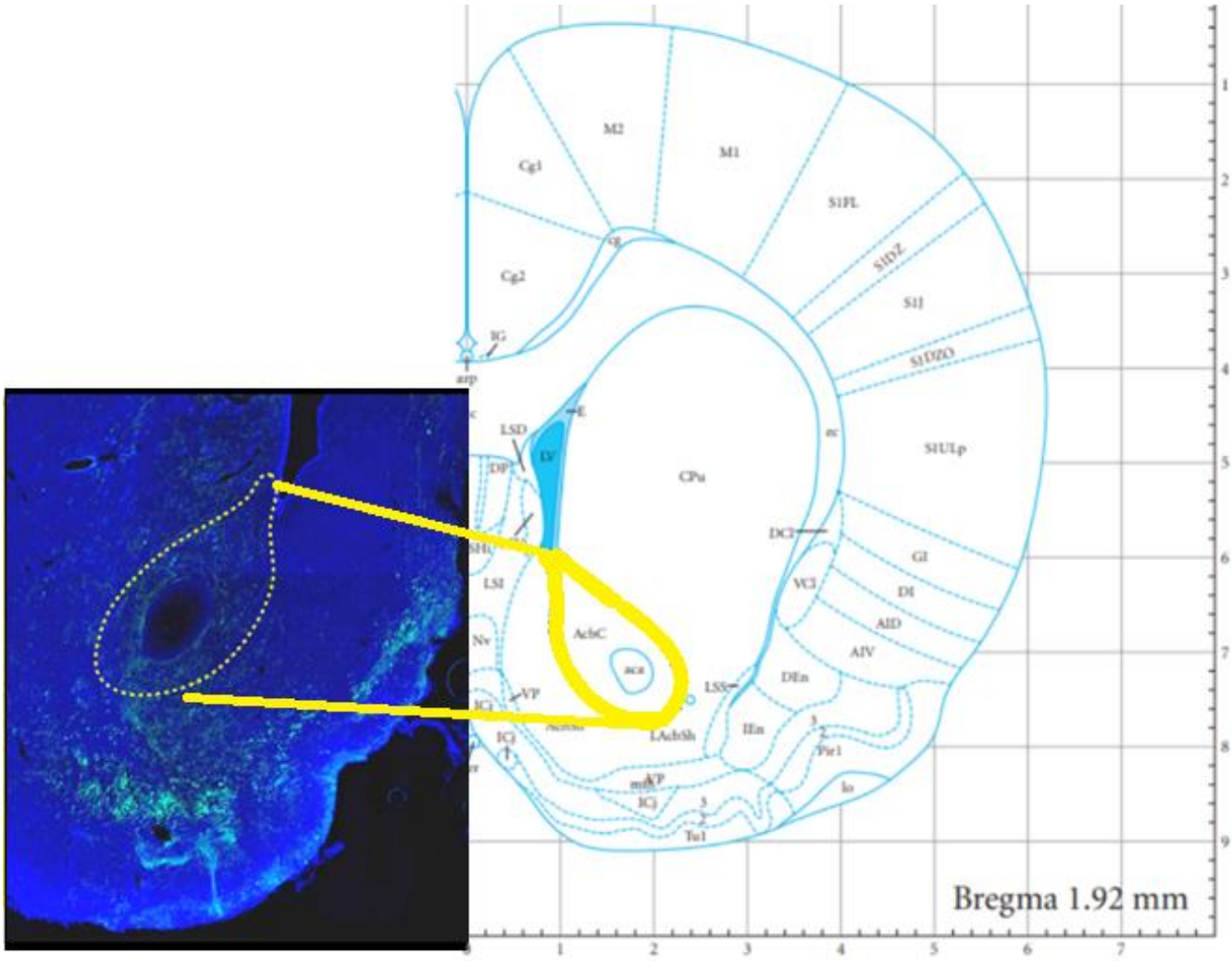


Figure 4.5.iv Zs Green Retrograde Expression (from VP to NAc)

Exp. 1: Self-Administration Training Data

Over a period of 10 days, 6 rats learned to self-administer .75mg/kg infusions. On average, they were able to perform 55 infusions and 70 lever presses. It's worth noting that inactive lever presses averaged less than 10 per day. In a subsequent cohort, 10 rats were trained over 3-hour training sessions. These rats averaged 35 infusions and 45 active lever presses. Interestingly, inactive lever presses remained under 5 on average.

Exp. 1: Exp. 1: No Significant Effect of hM4Di-Mediated Inhibition of Ventral Striatopallidal Projecting MSNs on Demand Curves

After rats demonstrated acquisition of within-session threshold procedure, 6 GFP rats and 2 hM4Di rats were treated with Vehicle, and 4 GFP and 4 hM4Di rats were treated with CNO. Demand curves were generated using publicly avaiable Prism templates "The Exponential Model of Demand (Hursh & Silberberg, 2008)" (http://ibrinc.org/software). No significant difference was found between Q0 values ($F_{(3,35)} = 2.4$, $p=0.0814$) or alpha values ($F_{3,35}=2.1$, $p=0.1198$) . Figure 4.5.vi is a bar graph depicting the average values of Q0 and alpha for each group (left and right, respectively). Green bars represent GFP-expressing rats, grey bars represent hM4Di rats, solid bars represent vehicle treatment, and slashed-bars represent CNO treatment. The unfitted average demand curves are shown in Figure **4.5**.vii (top panel). Green lines represent the GFP group, grey lines represent the hM4Di group, solid lines with solid shapes represent the vehicle groups, and dotted-lines with empty shapes represent the CNO treated groups. Individual demand curves for GFP and hM4Di subjects shown below the group averages. Solid green lines represent the GFP-Vehicle subjects, dashed yellow lines reresent the GFP-CNO subjects (Figure **4.5**.vii bottom left). Solid grey lines represent the hM4Di-Vehicle subjects and dashed yellow lines represent the hM4Di-CNO subjects (Figure **4.5**.vii bottom right).

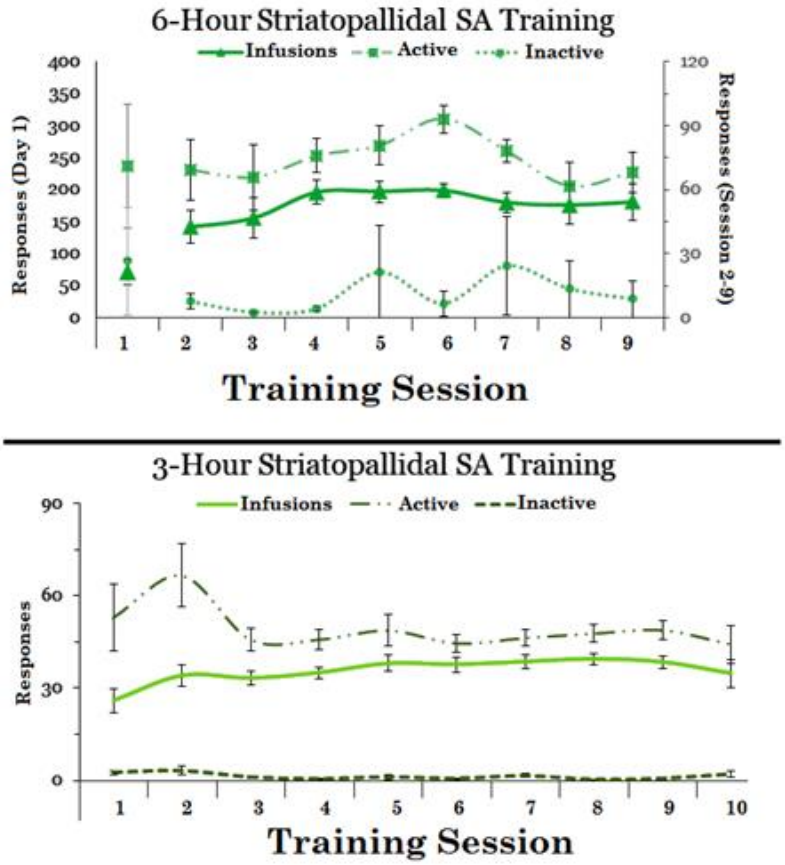


Figure **4.5**.v SA Training Data: Within-Session Threshold Cohorts

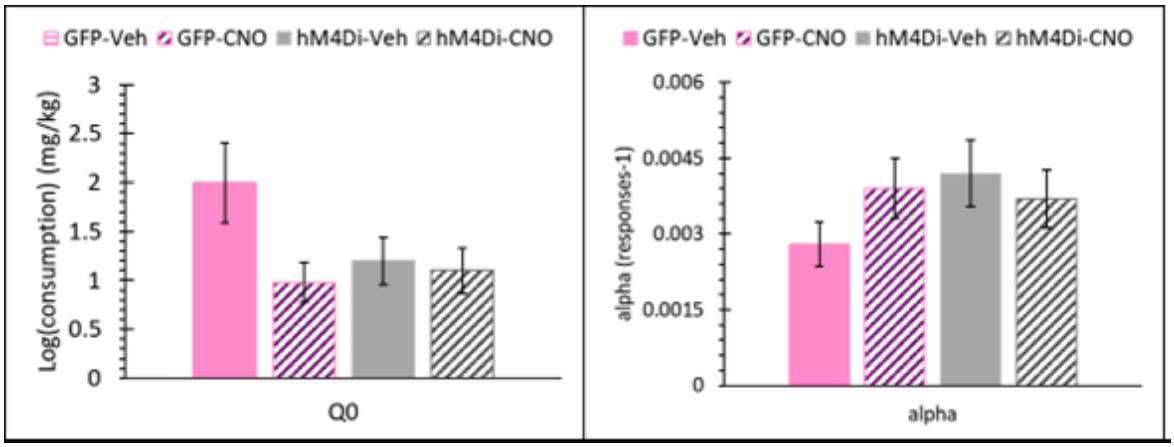


Figure 4.5.vi No Effect of CNO on Q0 or alpha

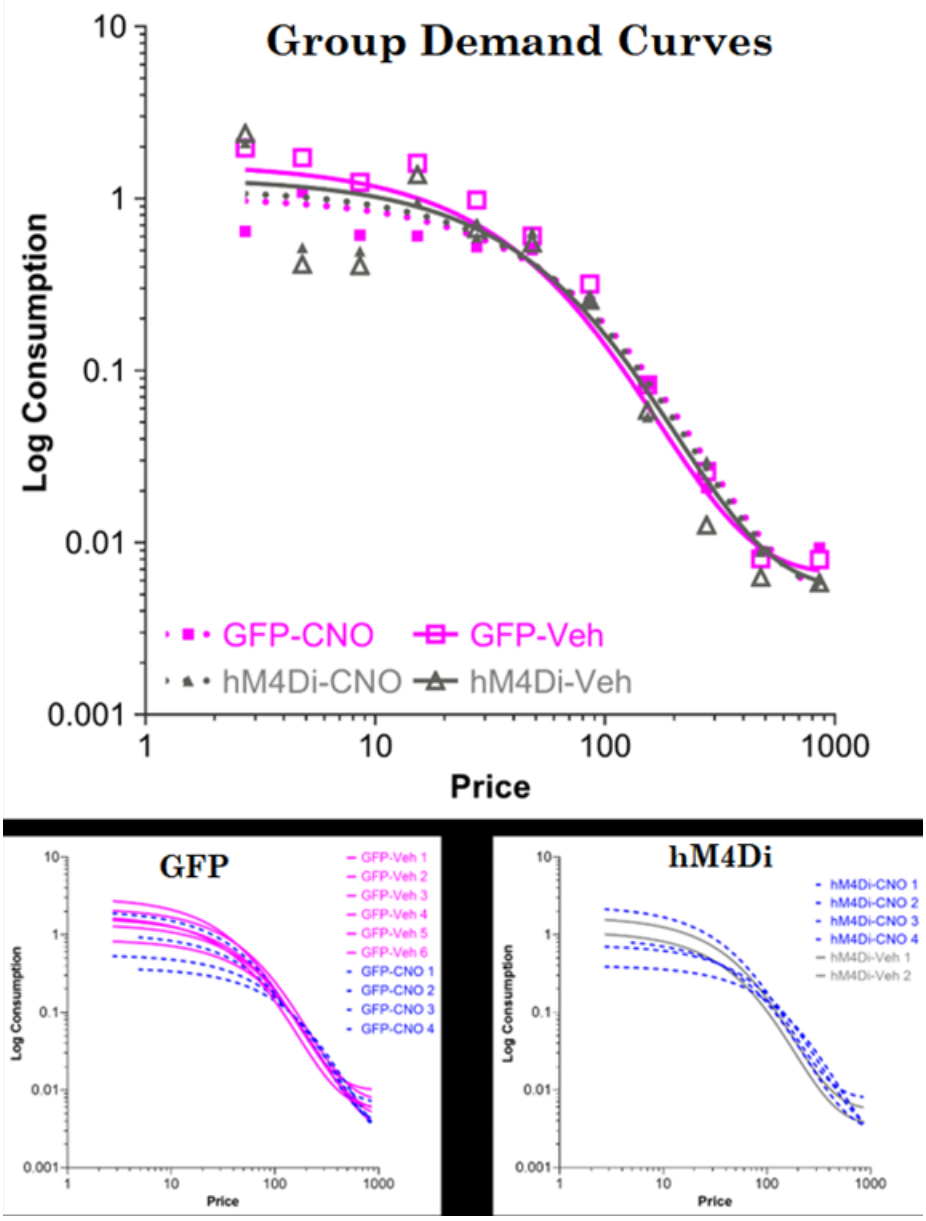


Figure **4.5**.vii No Significant Difference Between Group Demand Curves

4.6 EXPERIMENT 2: INCUBATION OF CRAVING RESULTS

Exp. 2: Increased HA-Expression in Transgenic Rats

RiboTag expression can be visualized by immunohistochemistry for HA-expression in the NAc core and confirmed via immunohistochemistry. Figure **4.6.viii** shows the confined

expression of HA within the NAc Core. Unlike in experiment 1, HA is a direct measure of RiboTag because the RPL22 are individually triple-HA-tagged. In contrast, the Zs-Green is an indirect estimate that only controls for the accuracy of retrograde viral infection and not cre-dependent viral expression.

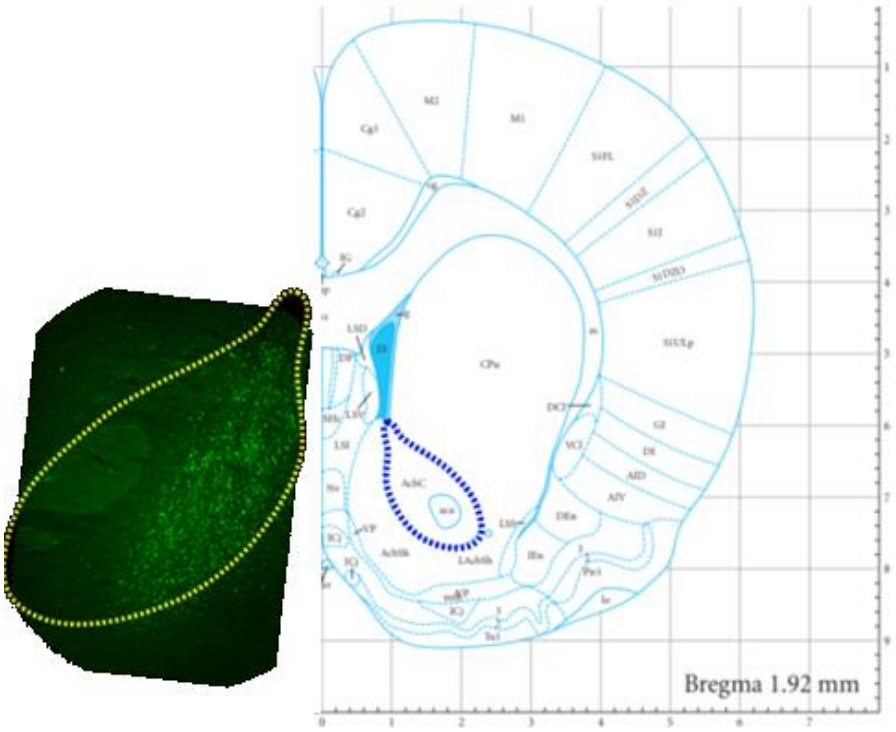


Figure **4.6**.viii DIO-hM4Di-RiboTag HA-Expression Validation

Exp. 2: Self-Administration Training Data

13 A2a-Cre females, 14 A2a-Cre males, 11 D1-Cre females, and 18 D1-Cre males were trained to self-administer cocaine (Figure **4.6**.ix). Over a period of 10 days, 56 rats learned to self-administer 0.375mg/kg infusions. Female rats averaged 50 infusions and 103 ±15 active lever presses whereas male rats averaged 35 infusions and 52±14 lever presses. Interestingly, inactive lever presses remained under 17±8 on average for females and 8±9 on average for males. There was an interaction between sex and inactive lever presses and another for sex and active lever press. Female rats responded more than male rats in the first session and had a significant reduction in inactive lever presses after the second day of training ($F_{9,428}$ =2.079, p=0.03); in contrast, an interaction was found between active lever presses, session, and genotype; multiple comparisons showed it was due to A2a males rats responded less in the first few days and their active lever responses significantly increased after the second day ($F_{9,424}$ =1.917, p=0.048). Responding over the last four sessions when the drug availability was reduced had no immediate impact on self-administration data. However, moving forward a consistent training schedule will be important as many other groups have found minor differences in training regime to have large impacts on both drug consumption and subsequent drug seeking behaviors. There was no significant sex effect in the training data.

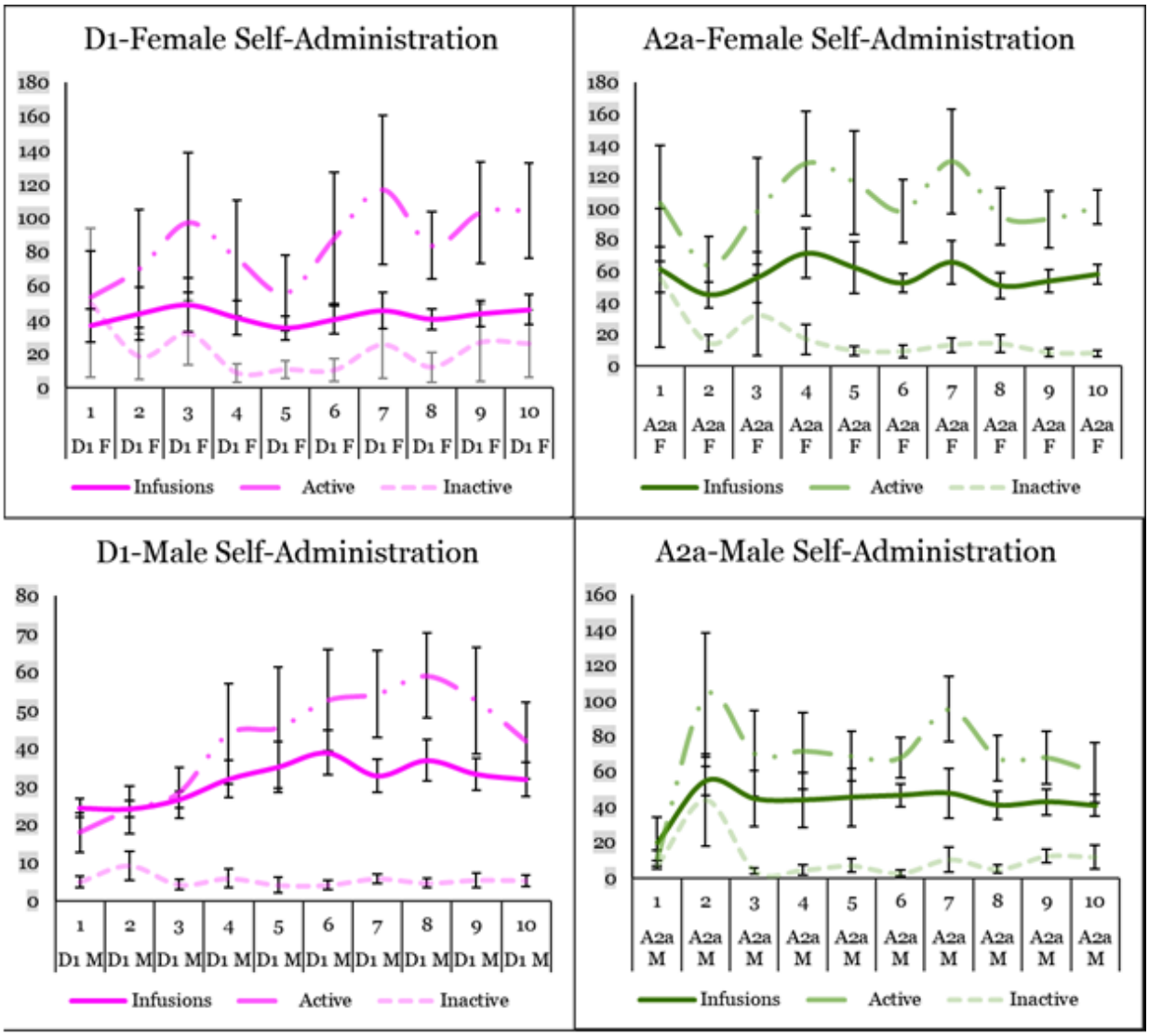


Figure **4.6**.ix Incubation of Craving: Self-Administratrion Training Data

Exp. 2: Inhibition ofct Pathway Blocks the Incubation of Cocaine Craving

After a period of forced abstinence, all but two group showed the hallmark incubation of craving (Figure **4.6**.ix). The results of a repeated measures ANOVA found a significant main effect of sex ($F_{1,67}$ = 4.268, p=0.043), and day ($F_{1,48}$ = 180.804, p<0.0001). It also found interactions between treatment and day ($F_{1,48}$ = 12.614, p=0.0009), sex and day ($F_{1,48}$ = 14.417, p=0.0004) and genotype, treatment, and day ($F_{1,48}$ = 20.469, p<0.0001).

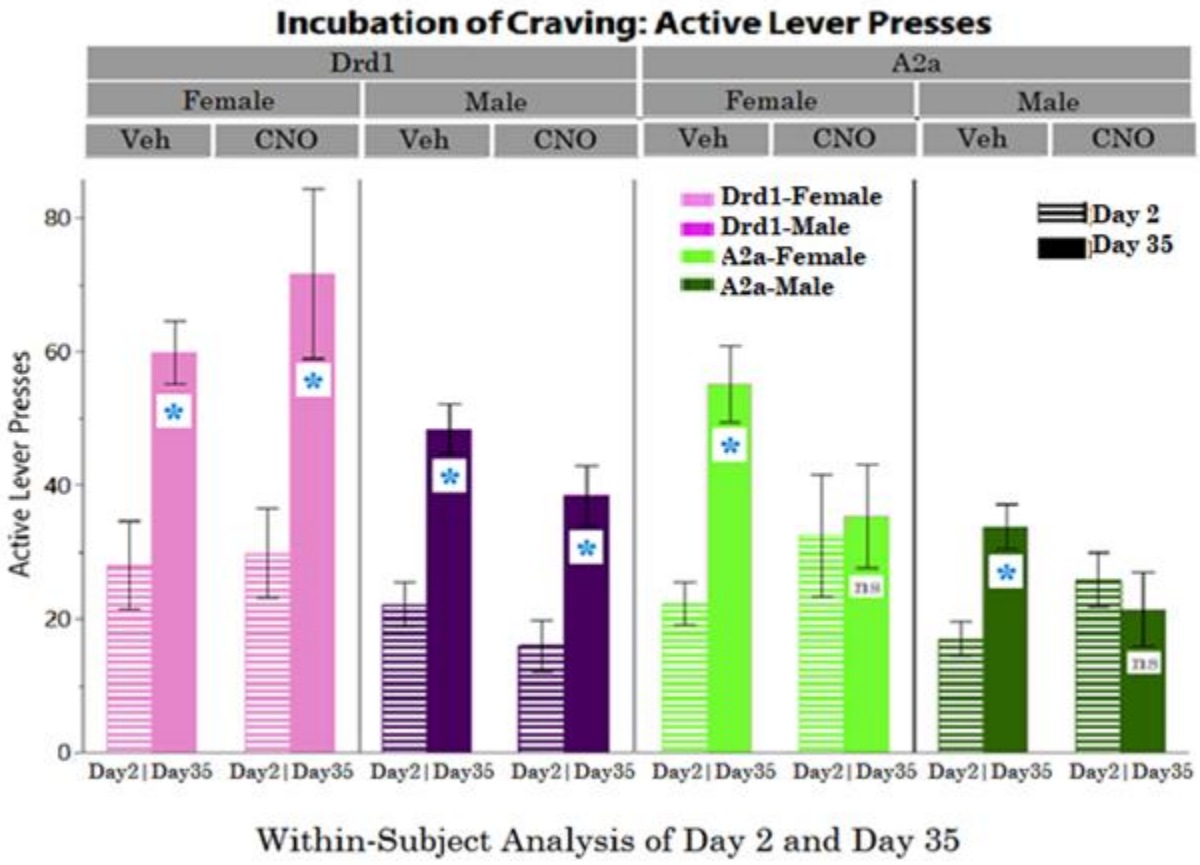


Figure 4.6.x Active Lever Presses: Day 2 vs. Day 35

Comparison of Day 2 and Day 35 of Forced Abstience demonstrating the Incubation of Craving in all groups except for A2a Males and A2a Females Treated with CNO

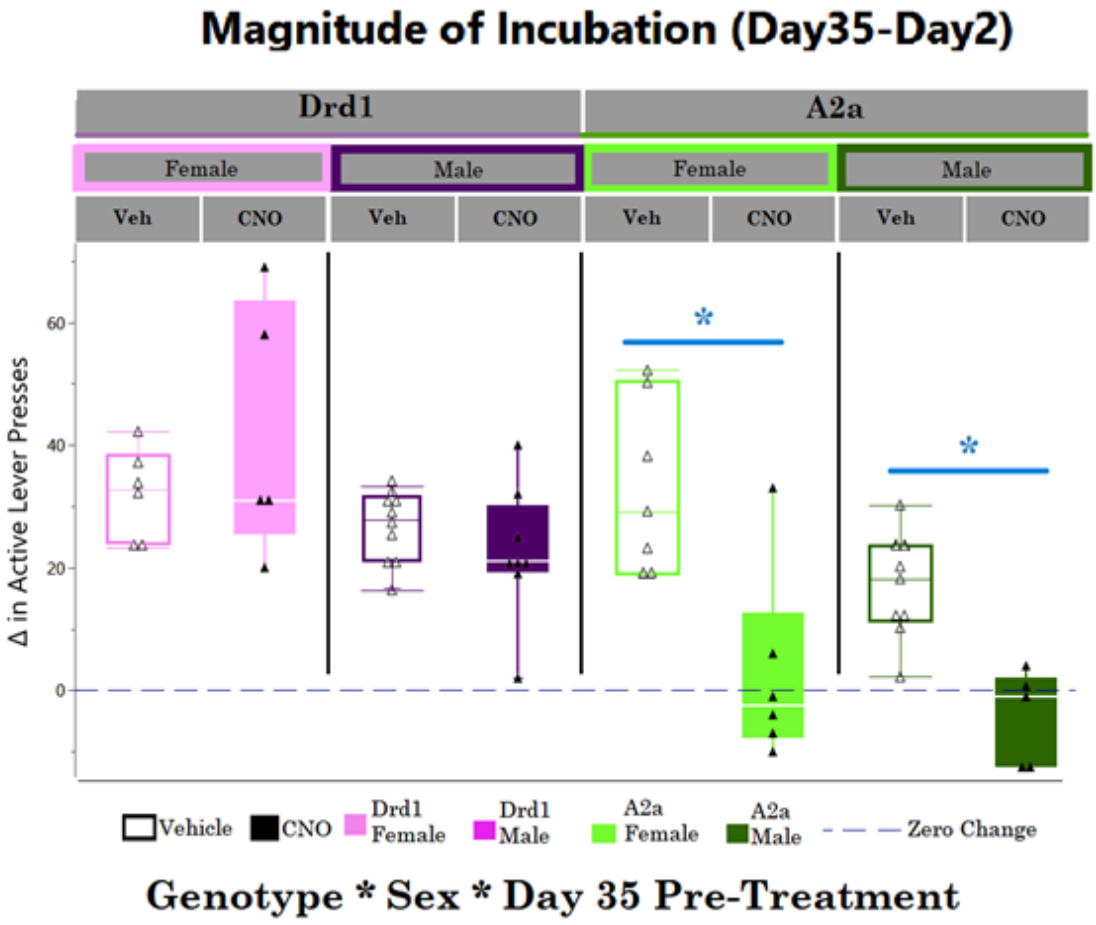


Figure 4.6.xi Difference in Responding Between Probe Tests (Day 35 – Day 2).

Among males and females, A2a inhibition resulted in a smaller increase in lever pressing between Day 2 and Day 35. Overall, female rats had a larger incubation effect than males based on the difference between the number of lever presses on test days.

Exp. 2: No Significant Effect of Incubation of Craving on 5-HT_{1B} Expression in ventral striatal D1 or A2a MSNs

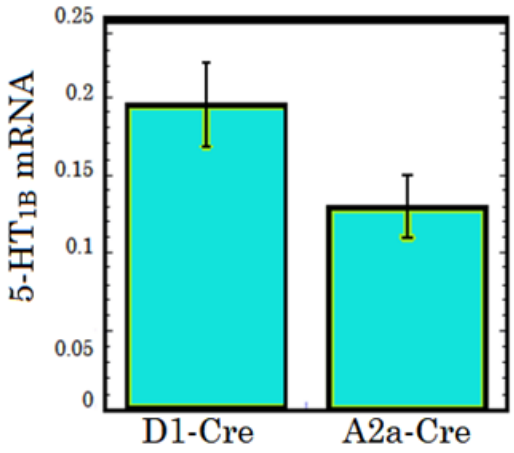


Figure **4.6**.xii No significant difference in 5htlB expression in A2a and Dl neurons after 35 days of forced abstience

Table 4.6.ii Table of Primers used for qPCR

HTR1Brat FWD Set 1	CACTGATGCGGTGGACTATT
HTR1Brat REV Set 1	AGCGAAATCGAGATGGAGAAG
HTR1Brat FWD Set 5	CGACGACTACATTTACCAGGAC
HTR1Brat REV Set 5	CCGATACACCGTAGCGATTAC

4.7 **Discussion**

Dynamic Role of Indirect Pathway Signaling on Drug Seeking

The behavioral results from these experiments support previous findings that the direct and indirect pathways dynamically regulate outputs to accomplish complex behaviors.

The coordination amongst the various regions involved further necessitates a thorough understanding of the interactions between dopaminergic, serotonergic, glutamatergic, cholinergic, and GABAergic signaling.

No Difference in 5-HT$_{1B}$ mRNA Expression Following Forced Abstinence

In our current study, there was no significant difference observed between 5-HT$_{1B}$ mRNA expression between the A2a or D1 neurons after 35 Days of abstinence. While 5-HT1B mRNA has been found to be regulated by acute stress (Fontaine et al., 2022) in a pathway-specific manner, and earlier studies found stress and cocaine exposure altered 5-HT1B mRNA in NAc (Liu et al., 2015; Neumaier et al., 2009; Pentkowski et al., 2012) at different stages, this does not appear to be the case after 35 days of abstinence. However, it is still possible that 5-HT$_{1B}$ signaling still can alter drug seeking at this delayed timepoint, but we did not examine this in these studies.

Future Directions

This can include RNASeq of some samples, qPCR probing of fractioned samples, time-course experiments to investigate temporal dynamics of serotonin expression during maintenance and abstinence.

4.8 References

Chapter 4

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CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

5.1 IMPORTANCE OF ADDICTION RESEARCH

Drug overdoses continue to plague the nation. With the increase in prevalence of synthetic drugs paired with the existing rates of poly-drug use and their combinatorial pharmacological effects (Crummy et al., 2020; Leri et al., 2004), specific approaches to successful treatments are likely necessary for people in different stages of addiction, from different demographic backgrounds, or with varying levels of resilience. Now more than ever, it is important to advance research on drug addiction to improve treatment outcomes. Chapter 1 describes only a fraction of the experimental strategies available to further develop our knowledge of addiction. More advanced techniques continue to be developed and integrated allowing for the field to tease apart even the most nuanced of questions. Our understanding of the behavioral and genetic changes resulting from direct and indirect striatal pathway activity could support a future where individualized treatment plans are available to target sex effects, subregional brain activation, cell-type specific activity, and nuanced differences in behavior. Previous evidence supports a sex effect on the vulnerability to relapse, suggesting females are more likely to suffer from more severe cravings and relapse potential (Lynch & Taylor, 2004); the root cause of this sex effect appeared to be estrous cycle dependent (Corbett et al., 2021; Kippin et al., 2005; Nicolas et al., 2019). On the other hand, a recent review of national data on drug use and mortality rates found that males are more susceptible to both drug misuse (*Key Substance Use and Mental Health Indicators*) and fatality (Butelman et al., 2023). Potential sex effects have direct relevance to treatment outcomes and further studies need to evaluate sex-specific drug effects on various self-administration models. Townsend et al. found

methadone treatment was effective in male rats but not in female rats (Townsend et al., 2021). It is unclear whether these sex effects are consistent in clinical studies, but the importance of understanding potential homology remains pertinent. Unlike for opioids and alcohol, there are no FDA-approved treatments for cocaine or other psychostimulant abuse or withdrawal. Better understanding differences in gene and protein expression between the sexes, and elucidating which projections and networks are responsible for specific behaviors could allow for treatment development that minimizes side-effects. Chapters 2, 3, and 4 describe the accumulate some of my work investigating the impact of stress on 5-HT1B expression, the roles of the LHb and NAc on reinstatement and relapse, and pathway specific dependency of the ventral striatum on the incubation of cocaine craving.

5.2 Role of 5-HT1B in Relapse and Reinstatement

The results in Chapters 2 and 4 move the field forward by describing how 5-HT1B expression specifically increases in Pdyn+ MSNs following forced swim, how inhibition of the indirect pathway is mitigates the incubation of craving following short-access self-administration training, and how 5-HT1B levels might not differ between the direct and indirect pathway. The novel aspects of these studies include the use of highly sensitive mRNA probes in RNAScope, the use of cell-type specific viral expression in behaving transgenic rats, and the potential for discovering changes in abstinence-induced mRNA expression. RNAScope probes can be multiplexed and are both sensitive and selective for their targets; this allows for quantification of colocalized mRNA in individual cells. Typical antibodies for either in-situ or immunohistochemistry fail to achieve clear and

abundant staining for the direct or indirect pathway MSNs (Pdyn, A2a, D1, Enk), so the methods used in this research are more definitive than previously used strategies.

5.3 Sex Effects or Something Else?

Although it is becoming more common and even a requirement for some funding agencies, sex as a biological variable can be challenging to implement depending on the specifics of the experimental design. The behavioral economics experiment in Chapter 4 was designed to investigate the role of the direct and indirect pathways on within-session threshold demand curves. The novelty of this approach includes the use of intersectional viral surgeries for pathway characterization and, the ability to assess multiple drug concentrations within a single 110-minute session. This model can be implemented repeatedly in a short time frame making it ideal for studying the effect of pharmacological treatments on dose-dependent responses to reinforcers and demand curves. Furthermore, it allows for investigating the motivation for drug consumption and any synergistic effects that occur while cocaine is in the system at their preferred level. It is comparable in nature to progressive ratio tests and serves as another tool in the arsenal of techniques available to study the motivation for drug taking – as opposed to drug seeking in reinstatement and relapse tests. However, due to extenuating circumstances, the study was first modified to investigate the effect short-access self-administration models motivation and drug-level preferences, and then further restricted to the indirect pathway to reduce animal usage. The changes in behavioral training schedules are problematic because of documented effects of increased or extended training (Kawa et al., 2016), intermittency of reinforcement (Zimmer et al., 2012) and drug valuation (Kazan et al., 2020). Additionally, it would be interesting to include sex as a biological variable

because of discrepancies that exist in the current literature regarding behavioral economics (Powell et al., 2019). Therefore, the within-sessions results presented in Chapter 4 should be viewed as preliminary.

5.4 Pathway Specificity in the Nucleus Accumbens

This dissertation includes a comparison of genotype and sex in rats that self-administered cocaine, were tested on the incubation of craving, and their brains processed for mRNA analysis throughout Chapter 4. This is particularly important as the exploratory nature of RNAseq has identified sex-specific effects in the translatome. Unfortunately, these samples will not be processed for RNAseq because the level of mRNA obtained per group was insufficient. Multiple changes in self-administration parameters contributed to the dispersed sample size. However, the RNA that was obtained through RiboTag can be repurposed to study changes in mRNA expression of targeted genes using qPCR. One of our initial pilot studies suggests that no changes in 5-HT_{1B} occurred following extended forced abstinence. It is worth noting that due to low sample sizes with significant yield and a hesitance to send samples for sequencing, our current qPCR included a limited number of subjects that were grouped by genotype, including males and females. This precaution was taken to ensure rigorous approaches with consistent methods were used as to capture any potential subtle effects.

5.5 Conclusion and Future Directions

Although my intent of exploring changes through RNAseq fell short, there is still an opportunity to assess specific changes in a targeted fashion using qPCR. Preliminary analysis of 5-HT1B in brains from Day 35 did not show a difference in 5-HT1B expression

between A2a and D1 expressing neurons. The inclusion of other variables could make clear whether, and if so when, any changes in 5-HT1B expression occurred in the ventral striatum. It would be helpful to include a control group with no cocaine exposure. Saline could serve as a neutral reinforcer to see the effects of cocaine on 5-HT1B during acquisition, maintenance, early withdrawal, and late abstinence as it controls for the operant procedures. It would also be important to assess amongst cocaine-consuming animals how 5-HT1B expression changed over the several time points tested behaviorally. It is likely there is a reduction of 5-HT1B mRNA in D1 and/or A2a MSNs in the early stages of abstinence, particularly if animals find that time stressful (Fontaine, 2022). Similar to the experiment in Chapter 2, RNAScope in-situ hybridization could be used to quantify and compare 5-HT1B expression in rats during cocaine self-administration and following multiple lengths of abstinence. Also, it would be interesting to test the effect of stress-induced relapse on 5-HT1B expression and compare it specifically to cue induced reinstatement and cocaine-primed reinstatement. As we found in Chapter 3, it is possible for Gi-signaling to affect one particular type of reinstatement over another. In our case, hM4Di signaling in the LHb reduced cocaine-primed relapse, but not cue-induced relapse. RNAscope technology has recently advanced to allow for staining tissue sections multiple times with up to 4 probes each set. Theoretically, they predict up to 50 mRNA probes could be assessed on the same sections allowing for unprecedented localization of where mRNA is expressed.

In conclusion, my dissertation research contributes to the field of neuroscience by advancing our knowledge of pathway specific changes that occur in response to stress and prolonged abstinence. Specifically, Chapter 2 found that 5-HT1B expression in the NAc of

mice was temporally regulated within Pdyn+ MSNs. Chapter 3 found that inhibiting the LHb enhanced cocaine self-administration, but subsequently mitigated cocaine-primed and cue-induced reinstatement. Chapter 4 found that inhibiting A2a MSNs in the NAc after 35 days of abstinence blocked the incubation of cocaine craving. These findings together demonstrate that continued dissection using fine-tuned approaches is necessary to understand the underlying neurocircuitry of stress and addiction. They highlight the importance of the ventral striatal direct and indirect pathways in responding to stress and encoding the vulnerability for relapse. Multiple regions share responsibility for the totality of any behavior, so understanding how the contribution of these subregions interact within a network (i.e., how the LHb and NAc regulate dopamine and serotonin) will be critical. The timing of activation remains noteworthy, and dual approaches that increase temporal and regional precision will further delineate regional roles and contributions.

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Chapter 5

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