The Functions of Cholinergic Neurotransmission in the Nucleus Accumbens

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Abstract

The nucleus accumbens (NAc) has long been regarded as essential brain structure for learning and motivated behavior. Acetylcholine (ACh), from local cholinergic interneurons, comprise a significant source of neuromodulation in the NAc. Though how these cholinergic interneurons function in the striatum is not fully understood, they have been shown to encode both salient and reward-related stimuli and have been implicated in supporting certain forms of learning. This thesis attempts to improve our characterizations of this neuromodulatory population and expand our understanding of its role in learning and behavior. In the case of cholinergic interneurons, while recent work has demonstrated the necessity of cholinergic transmission to reward-context learning several gaps remain in our understanding of this neuromodulator. A causal role for cholinergic activation, for instance, has not been convincingly shown in the striatum for Pavlovian reward learning and acetylcholine’s relationship to neural plasticity is not well characterized. Using optogenetic approaches to resolve these problems, we demonstrate how striatal cholinergic activation influences reward-context learning. Using ex-vivo electrophysiology, we then examine how cholinergic mediated changes in learning correspond to synaptic plasticity. These are the first findings that convincingly link changes in cholinergic activation with learning and plasticity.
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Organization of the Thesis

This thesis presents results of work undertaken to further our understanding of how cholinergic interneurons in the striatum contribute to learning and behavior. We outline how cholinergic neurons contribute to reward context learning and their relationship to plasticity and reinforcement. In Chapter 1, I begin by reviewing background information on learning and behavior in the brain and then examine findings on the role of cholinergic transmission in learning and plasticity. I then proceed to outline some of the pressing question that existing findings fail to address and motivate our goals in this thesis. In Chapter 2, I introduce the conditioned place preference task, a key behavioral paradigm I employ to examine context learning in mice for the duration of the thesis. Here, we examine the role of cholinergic interneurons in extinction learning of a cocaine-context association and display that they confer bidirectional control over cocaine extinction learning. Given its role in cocaine-context extinction, I next seek to extend this finding to other types of reward extinction learning in chapter 3; for food and fear context associations. We find that cholinergic activation, surprisingly, fails to influence these other forms of context-learning. We conclude this chapter by showing that cholinergic interneurons fail to reinforce behavior by themselves, suggesting they play a modulatory role in learning. In Chapter 4, we interrogate why cholinergic interneurons can uniquely modulate cocaine, but not other forms of context
extinction learning. We hypothesize that since cholinergic activation plays a modulatory role in learning, it may play a modulatory role in facilitating underlying plasticity. We thus begin by demonstrating, using ex vivo electrophysiology, that such plasticity indeed occurs during cocaine context extinction. We then show that cholinergic activation enhances this plasticity in the NAc as a mechanism to support changes in behavior. We show these changes are context dependent and require the co-occurrence of ongoing learning to effect plasticity. We therefore conclude in chapter 5 that cholinergic interneurons serve a modulatory, rather than causal role in reinforcement and plasticity. We tie this finding into existing literature on reward and plasticity in the NAc and examine the ramifications of these findings for conceptions of cholinergic interneuron functionality and its intrinsic activity. We complete our concluding chapter by outlining suggested next steps for future research.
1 Introduction

1.1 Learning and Behavior

In order to survive, organisms must be able to learn reward associations and they have to implement decisions and execute behavior to take advantage of the associations they have learned. These abilities collectively enable organisms to locate, track, and obtain food or sexual rewards, often in changing and complex environments. Reward learning and implementing decisions and behavior are closely related function in the brain but are typically conceptualized to occur through distinct processes and in different phases.

Reward learning pertains to a procedure through which organisms acquire information regarding contexts, actions and stimuli that predict valuable results. Reward learning is demonstrated when an organism modifies behavior in response to either a novel reward or unexpected changes in reward outcomes and is commonly subdivided into different phases. First, in a simple Pavlovian framework, acquisition occurs when a conditioned stimulus (CS), a neutral stimulus such as tone or neutral context, is presented, in close proximity, to an unconditioned stimulus (US), such as sucrose or drug, which carries latent reward-value. Learning of a reward association is considered acquired when the CS produces a response to the previously neutral conditioned stimulus, formerly elicited only by the US, a conditioned response. Next, extinction occurs when the
CS is presented repeatedly without the US, which leads to the reduction of the conditioned response.

These reward associations however, are useful to animals only if they can implement decisions and execute complex behaviors to take advantage of what they have learned. Organisms must spatially represent and navigate demanding environments, engage in complex locomotion, anticipate and attend to competing stimuli and select actions predicated on preferred outcomes with incomplete information.

1.2 The Nucleus Accumbens: Roles in Learning and Behavior

In the brain, many essential components of both learning and behavior are subserved by the nucleus accumbens (NAc). The NAc plays an especially important role for selecting actions and integrating affective and cognitive inputs from temporal and frontal areas to motivate aversive or appetitive behaviors. In the NAc, further divided into shell and core sub regions, inputs arrive from a wide swath of upstream regions including the ventral hippocampus (V.hip), prefrontal cortex (PFC), basolateral amygdala (BLA), thalamus and VTA. Activation of NAc inputs from any of these regions is sufficient to reinforce behavior (Britt et al., 2012; Zhu et al., 2016). Each region computes distinct categories of information which inform dissociable behavior patterns relevant to reward through interactions with the NAc. Projections from the V.hip, to NAc shell for example, are implicated in mediating reward foraging behavior in complex environments and navigating during discriminative approach via reward-related cues ( Seamans and Phillips, 1994; Ito
et al., 2008). Inhibition of the BLA and its glutamatergic afferents, by contrast, impair reward consumption and motivation (Stuber et al., 2011), while PFC interactions with the NAc appear to impact behavior requiring high attentional demands (Christakou et al., 2004), planning (Block et al., 2007) matching behavior to context (Floresco and Phillips, 1999), mapping of social reward to space (Murugan et al., 2017) and deliberating cost/benefit evaluation (Hauber and Sommer, 2009). These synaptic inputs to the striatum terminate largely onto medium spiny projection neurons (MSNs), an inhibitory, gamma-aminobutyric acid (GABA) expressing cell-type which comprises over 90% of all cell-types in the striatum.

Taken together, these attributes position the striatum to integrate incoming information in order to both learn to perform reward yielding behaviors and then generate the behaviors it has learned to perform. Firing patterns of NAc neuronal populations, for example, both signify reward predicting activity and can bias in motion directions or alter behavior through outputs to other basal ganglia nuclei which exert control over motor activity and kinematics.
A causal role for these NAc reward-responses in learning for instance, has been demonstrated in several studies. These studies showcase how NAc lesions, and alteration of NAc dopamine, hinder the expression as well as acquisition reward-related behavior (Blaiss and Janak, 2009; Everitt et al., 1991; Nicola, 2010; Parkinson et al., 2000; Saunders and Robinson, 2012). The NAc has also been characterized to play an essential role in transforming reward cues into incentive salience, driving approach toward cues, aiding in discriminative approach and invigorate ongoing reward seeking (Christakou et al., 2004; Corbit and Balleine, 2012).
2011; Corbit et al., 2001; Maldonado-Irizarry and Kelley, 1994; Mogenson, 1993; Pezze et al., 2007; Wu et al., 1993). A few studies also suggest the NAc is also involved in the acquisition of contextual Pavlovian fear responses (Haralambous and Westbrook, 1999; Levita et al., 2012; Parkinson et al., 1999), and discrete fear-cue learning as well (Oleson et al., 2012).

Taken together, this evidence portrays the role of the NAc not as a limbic motor interface as such, since NAc lesions fail to abolish motor behavior, but rather as a nucleus that expediates approach toward reward-associated contexts or stimuli (Khamassi and Humphries, 2012; Mannella et al., 2013; Nicola, 2007). Glutamatergic inputs to the NAc, dictate appropriate response priorities in this conception, but learning requires that these inputs have some means of modulation to strengthen the most appropriate neural repertoires and suppress unrewarded ones.

1.3 Neuromodulation in the Striatum in Learning and Behavior

Theories of how the striatum contributes to reward learning, almost universally rely on the input of neuromodulatory transmission and resulting changes in synaptic plasticity which impact glutamatergic inputs. Neuromodulatory changes in synaptic connections are thought to provide a crucial mechanism for consolidation of learning to guide behavior over long time scales. The way these neuromodulators play a key role in the learning process in the NAc is by modulating the transfer of neural signals to alter the strength of synapses between MSNs and
incoming glutamatergic inputs. This serves to reinforce goal-oriented, complex behaviors (Cepeda and Levine, 1998). Acetylcholine comprises a prominent neuromodulatory striatal input which, like dopamine, is thought to play a critical role in synaptic plasticity in the NAc. While much work has focused on the role of dopamine, the role of cholinergic interneurons, the primary source of acetylcholine in the NAc, remains less well understood for learning and behavior. Our goal in this thesis is to further elucidate the role of this important population on specific modes of learning and shed a light on its ambiguous role in plasticity in the NAc. We begin the next section with a principal overview of historical and current findings on this important neuromodulatory population.

1.4 Acetylcholine Transmission in Learning and Reinforcement: Neural Correlates, Pharmacological Findings, and Causal Manipulations

1.4.1 NAc Cholinergic Interneurons, Physiology and Neural Correlates

Though their exact function in reward behavior and reinforcement is not well understood certain physiological features and their unique anatomy suggest an important role for cholinergic interneurons in NAc circuitry (Contant et al., 1996; Ligorio et al., 2009; Tepper and Bolam, 2004; Zhou et al., 2002). Anatomically, for instance, though they comprise anywhere from 1-5% of total neurons in the NAc (Descarries et al., 1997; Descarries and Mechawar, 2000; Rymar et al., 2004), cholinergic interneurons have been shown to play a critical role in orchestrating...
circuit activity. They drive local release of dopamine (Cachope et al., 2012; Threlfell et al., 2012; Zhou et al., 2001) and control the output of MSNs (English et al., 2012; Oldenburg and Ding, 2011; Witten et al., 2010; Nelson et al., 2014; Higley et al., 2011).

Like VTA dopaminergic neurons, recordings from putative cholinergic interneurons (often referred to as TANs or tonically active neurons in this literature) show these neurons to report unexpected rewards in addition to environmental cues that predict reward (Aosaki et al., 1995; Benhamou et al., 2014; Morris et al., 2004; Wilson and Rolls, 1990; Zhou et al., 2002) as well as rewarding contexts (Atallah et al., 2014; Apicella, 2007) and tracking environmental states about reward availability (Stalnaker et al., 2016). These responses to reward, like dopamine, adapt to experience and change over the phases of reward learning (Atallah et al., 2014). And further, like dopamine neurons, putative cholinergic interneurons in the NAc exhibit unique bidirectional outcome responses, not merely responding to rewards with phasic excitation, but also displaying inhibition in response to reward omission (Atallah et al., 2014). This activity profile of TANs as neural correlates of putative cholinergic interneurons in the NAC obviously portrays a parsimonious fit for learning signals given the above findings. Nevertheless, some caution is required with interpreting these neurophysiological data as recent findings demonstrate spontaneous tonic activity in the NAc in both parvalbumin positive interneurons and nitrous oxide-releasing interneurons (Beatty et al., 2012; Berke, 2008; Sharott et al., 2012). This raises significant
challenges for cleanly interpreting previous physiological findings because it suggests that in some cases, the tonically active neurons being recorded may not have been cholinergic interneurons after all. To resolve the outstanding question regarding neural correlates of cholinergic interneurons during behavior requires better specification for these correlates, through genetically encoded calcium indicators for example.

1.4.2 NAc Cholinergic Interneurons, Pharmacology and Transmission Studies

Pharmacological studies confirm a critical role for acetylcholine transmission in the NAc in learning and motivation for both drugs and natural reward. Numerous studies for example, demonstrate that interference in muscarinic and nicotinic receptor activity in the NAc blunt instrumental or context learning for reward behaviors (Crespo et al., 2006; Hikida et al., 2003; Itzhak and Martin, 2000; Mark et al., 2006; Rasmussen et al., 2000; Thomsen et al., 2010) and acetylcholine levels escalate in this region in direct response to both food and drug reward-consumption (Avena et al., 2006; Berlanga et al., 2003; Mark et al., 1999; Mark et al., 1992; Rada et al., 2005;). Some evidence suggests this elevation in NAc acetylcholine actually causes satiety for both food and drug consumption. Infusion of acetylcholine receptor (nAChR) agonists, which principally effect the NAc, (Feduccia et al., 2014) causes reduced sucrose intake in a dose dependent fashion (Shariff et al., 2016) for instance, and nAChRs
agonism and antagonism both inhibit drug consumption and lever pressing respectively during self-administration (Mark et al., 2006; Thomsen et al., 2010). Taken together, these reports strongly suggest that acetylcholine transmission, in the NAc specifically, plays an essential role in learning and serves as a consummatory signal which may itself cause satiety for both food and drug reward. There are several caveats with this research. Pharmacological agonism and antagonism in nAChRs can yield identical effects on downstream dopamine release for example, and these interventions fail to replicate the endogenous and highly complex profile of cholinergic interneuron physiology. Causal research is thus needed to clarify the precise role of cholinergic interneurons, but only a handful of studies have probed causal roles for stimulation of cholinergic interneurons.

1.4.3 NAc Cholinergic Interneurons, Causal Manipulations

Amongst the few studies that have probed a causal role for cholinergic interneurons, most show effects over various forms of learning and cognition. These effects are widely diverse, with downstream cholinergic interneuron activation enhancing discrete cue-fear associations, cholinergic interneuron inhibition blocking cocaine-context acquisition and also enhancing fear-context learning (Brown et al., 2012; Witten et al., 2010). Furthermore, recent evidence shows that chemogenetic activation and inhibition of cholinergic interneurons yields bi-directional control of food consumption (Aitta-aho et al., 2017). Some lesion studies provide evidence for this role in learning, as specific immuno-toxic
lesions on cholinergic neurons alter learning within in an attentional set-shifting (Aoki et al., 2015) and others show selective NAc lesions feeding stereotyped and fixated social behaviors (Martos et al., 2017). Collectively, these studies offer important insights into the function of cholinergic interneurons and convincingly tie the acute activity of these neurons to many of the operations that pharmacological and neural correlate studies suggest.

Yet several limitations have prevented a more comprehensive understanding of the functional role of cholinergic neurons: What is the exact relationship between cholinergic stimulation and reinforcement? Do they instruct new learning like dopamine neurons do? Does cholinergic activation cause plasticity and if so under what conditions? These questions provide significant gaps in our collective conceptions of how cholinergic interneurons support learning and behavior.

1.5 Motivation

1.5.1. The Role of Cholinergic Interneurons in Cocaine Context Extinction

Here, we compare the role of cholinergic interneurons in several types of context extinction learning, focusing especially on cocaine-context associations. We begin by running mice on a series of context association extinction tasks (see chapters 2-4) that train animals to associate various reward or fear stimuli to
specific contexts and test how well they have learned the context-association after training. We use the behavior of the animals, time spent in reward-associated contexts vs neutral ones, and freezing in fear associated ones, as a readout of learning, specifically measuring the learning of an extinction-context, since the US is no longer present. We employ cocaine and food as rewards in a conditioned place preference paradigm and deliver shocks in a conditioning chamber as a context for fear learning.

Competing concepts of striatal cholinergic neurons’ role in altering reward-association suggest distinct outcomes for how and when altering cholinergic activity will influence the learning of a reward extinction context. We have described, for example how acetylcholine transmission in the NAc has been shown to alter reward behavior by imposing a satiety signal (Avena et al., 2006; Avena et al., 2008; Pratt and Kelley, 2004; Feduccia et al., 2014) and chemogenetic manipulation of cholinergic interneurons yields bidirectional control of food consumption (Aitta-aho et al., 2017). But cholinergic interneurons also display reward prediction error signalling and evidence suggest they are necessary for reward learning broadly and cocaine-context acquisition specifically (Witten et al., 2010). A critical question thus emerges that brings previous findings into sharp relief: Is cholinergic manipulation in the NAc interfering with cocaine CPP by disrupting cocaine-environment association learning or by decreasing the hedonic components of cocaine reward? The existing evidence clearly supports roles for acetylcholine transmission both in learning and satiety and thus an experimental
approach is needed to better segregate these explanations. Cocaine context extinction comprises a highly suitable learning paradigm to draw this distinction because we intend to stimulate cholinergic interneurons during a first extinction test, but not subsequent ones, in order to measure persistence in learning over time. If we witness persistence in context-preference changes in extinction tests run subsequent to cholinergic manipulation this indicates that learning, not satiety, supports these changes in behavior. Using optogenetic interventions in various behavioral paradigms, we hope to illuminate how cholinergic interneurons of the NAc contribute to reward-context learning and either validate or reform these current conceptualizations.

1.5.2 Linking Cholinergic Interneurons to Behavior and Plasticity

Another aim we wish to motivate in the course of this work is to clarify how NAc, acetylcholine transmission alters reward learning and its precise role in driving plasticity. If we find that manipulating these neurons modulates learning, could neural plasticity play a role and if so, can we characterize the conditions under which such plasticity occurs? This question comprises a substantial gap in our understanding of this population relative to DA, where clarifying how its neurotransmission influences plasticity, learning and behavior has proven so fruitful. Our research on this question aims to distinguish between two possible hypotheses. While it is possible that cholinergic activation, like DA activation is sufficient to induce reinforcement and plasticity on its own, cholinergic populations might instead serve to modulate gain on both learning and plasticity from ongoing
reinforcement. In order to better delineate how these neurons contribute to behavior and influence local computation, we aim to differentiate between these two possibilities. We sought to answer these and other questions by employing ex-vivo cellular physiological recordings to examine the effects of cholinergic interneuron-driven changes in plasticity in the NAc after context extinction-learning for food and fear context associations.
2. Cholinergic Interneurons Bidirectionally Control Extinction in a Cocaine Conditioned Place Preference (CPP)

2.1 Introduction

In this chapter, we introduce the Conditioned Place Preference (CPP) paradigm (citations), which comprises the key behavioral task employed in this thesis to study context learning. We will determine if cholinergic transmission contributes to changes in learning and plasticity in the context of extinction for a cocaine CPP. We chose cocaine as a reward both due to its efficacy as a reinforcer for reward-context learning and its obvious clinical relevance. Additionally, for clinical treatment of addiction, extinction remains an understudied, but important phenomena as addicts often persevere in habitual drug-seeking. Understanding the mechanisms that underlie extinction learning could therefore also yield potential clinical advances for diminishing habitual drug seeking behaviors. To manipulate cholinergic neurons, we will employ optogenetic tools in mouse genetic lines that provide us with precise temporal and spatial control over this population. By bidirectionally modulating the activity of these neurons during extinction learning, we hope to illuminate their role in context learning and key underlying mechanisms that support that role.
2.2 Methods

All procedures were performed in accordance with university-approved IACUC protocol.

2.2.1 Surgical Procedure

Animals were deeply anesthetized and we injected AAV2/5, Cre-dependent ChR2-YFP virus, NpHR-YFP or YFP-only (control virus) into the medial NAc (anterior-posterior (AP), 1.4 mm; medial-lateral (ML), ± 0.7–0.9 mm; dorsal-ventral (DV), 1 μl per site). of CHAT::IRES-Cre mice, a Knock-in line that express Cre recombinase only in cholinergic neurons to permit their unique viral targeting. We then implanted optical fibers bilaterally over the NAc We implanted optical fibers (300 μm core) at a 10° angles to target the medial NAc (AP, 1.4 mm; ML,0.7 mm; DV, 3.9 mm.)

2.2.2 Histology and Immunohistochemistry

After the completion of the experiment, animals were given transcardial perfusion with PBS followed by 4% PFA in PBS. Brains were subsequently transferred to 30% sucrose solution in PBS. 4 micron slices were then collected using a microtome for each brain. The relevant NAc slices were stained with DAPI and visualization was performed with a stereomicroscope (Leica) for slice imaging. For immunohistochemistry, we first washed 20 micron slices in PBS followed by
blocking for 30 minutes (0.3% Triton-X and 3% normal donkey serum (NDS)). We then incubated sections overnight in ChAT primary antibody (1:200; Millipore, product# AB144P [RRID: AB_2079751]) at 4°C in 3% NDS/PBS. After washing, sections were next incubated in secondary antibodies conjugated to AlexaFluor586 for 2 hours (1:1000; Life Technologies, product# A11057 [RRID: AB_10564097]). We next incubated with DAPI for 20 minutes (1:50,000), after which we washed and mounted slices for confocal imaging (Leica).

2.2.3 Ex-vivo Validation of Optogenetic Efficacy

Mice received a transcardial perfusion of ice-cold carbogenated NMDG ACSF (see Supplemental Experimental Procedures). After extraction, the brain was immersed in ice-cold NMDG ACSF for 2 min. Afterward, coronal slices (300 μm) were sectioned using a vibratome (VT1200s, Leica) and then incubated in NMDG ACSF at 34°C for 15 min. Slices were then transferred into a holding solution of HEPES ACSF (see Supplemental Experimental Procedures). During whole-cell recordings, slices were perfused with a recording ACSF solution (see Supplemental Experimental Procedures, Lee et al., 2016). Infrared differential interference contrast–enhanced visual guidance was used to select neurons that were 3–4 cell layers below the surface of the slices, which were held at room temperature while the recording solution was delivered to slices via superfusion driven by peristaltic pump. The pipette series resistance was monitored throughout the experiments; if the series resistance changed by >20% during the recording,
the data were discarded. Whole-cell currents were filtered at 1 kHz and digitized and stored at 20 KHz (Clampex 9; MDS Analytical Technologies).

### 2.2.4 Optogenetic Stimulation

For all ChR2 activation experiments, we measured 9–10 mW of power from the tip of implanted optical fibers for use of blue light (447 nm) and for all NpHR experiments, we measured 2-3mW of power from the tip of the implanted optical fibers for use of yellow light (590 nm). In all cases except for intracranial self stimulation (ICSS), we delivered light through an ON-OFF burst pattern (5 ms long pulses at 15 Hz for 2 s interleaved with 2 s light off periods). Yellow laser light was continuously for all NpHR stimulation experiments.

### 2.2.5 Cocaine Conditioned Place Preference

4-6 weeks after surgery, cohorts were conditioned on a cocaine conditioned place preference. We placed subjects in a 50cm x 30cm, two chamber arena separated by a central portal. The chamber’s walls were black, distinguished by horizontal lines on one side and vertical lines on the other. The flooring between the chambers was distinguished with bars on one side and mesh flooring on the other. These walls and floors were selected such that mice displayed no bias at baseline for either side of the apparatus. The CPP test was conducted as following:
On the first day, each mouse was placed in the central portal while connected to “dummy” patch cables that were not emitting light and allowed to freely explore the entire apparatus for 15 minutes (pre-test). Day 2 and 3 consisted of conditioning, wherein each mouse was confined to one of the side chambers for 20 minutes in the morning and then again, to the opposite chamber in the afternoon for an additional 20 minutes. Subjects received i.p. injections of cocaine (15 mg/kg) before placement in one chamber or i.p. injections of an equal volume of saline before being placed in the other chamber (0.1mL). Subjects underwent 3 extinction tests to measure and extinguish preference for the cocaine associated side. Day 4 consisted of the 1st extinction trial (Test 1) where mice received continuous optogenetic stimulation during the 15 minutes in which they had access to both chambers in the two chamber apparatus. On day 5 and 12 (Test 2 and 3), mice were again placed in the center chamber and allowed to freely explore both chambers for 15 min without stimulation (post-tests) (Fig 4).

![Figure 4](image)

Figure 4 - Cocaine conditioned place preference paradigm with optogenetic stimulation for activation and inhibition respectively on “Test 1”.

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2.2.6 Real Time Conditioned Place Preference

In real time conditioned place preference (RT CPP), animals were placed in a two-chamber apparatus described above with differing floors and walls. Each mouse was placed in the central portal while connected to patch cables and allowed to freely explore the entire apparatus for 20 minutes where they could freely move between sides. Light stimulation was paired to one chamber of the apparatus and remained on until the animal moved freely to the other side. Video tracking software was used to track mouse location and velocity.

2.2.7 Open Field Test

A chamber (100 × 100 cm) with a central open field was measured into a central area (center, 50 × 50 cm) and outer area (periphery). Subjects were positioned initially in the periphery and the animals were tracked video. Video-tracking software was used to analyze total distance traveled over space. The open field test was run the day after the elevated plus maze task. The test ran for a total of 5 minutes and in each session optogenetic manipulation was delivered throughout the whole test. In this task, proportion of time that subjects spend in periphery over the more exposed center serves as a measure of anxiety-like-behavior.
2.2.8 Elevated Plus Maze

The elevated plus maze (EPM) task occurred 2-3 weeks subsequent to the last extinction of the cocaine CPP task. The arena was constructed out of plastic and was made up of two light-floored arms (30 × 5 cm) in the “open” section, and two light-floored arms with black enclosures (30 × 5 × 30 cm) each arm emerging from a central cube (5 × 5 × 5 cm) and intersecting at 90 degrees in a symmetric, plus shape. The EPM arena was situated 50cm above the ground and at the start of the task, animals began by being placed in center. Before the session began, animals were permitted to recover from handling for 1-3 minutes. Software for motion tracking was used to monitor the mouse during behavior. Subjects ran the maze for 9 minutes, divided into 3 minute epochs, with blue or yellow light delivered for the entirety of the 2nd epoch. Time spent in the closed arms over the open arms of the EPM serves as a measure of anxiety-like behavior.

2.2.9 Intracranial Self Stimulation

Subjects were placed for 120 minutes in an operant chamber (Med Associates Inc.) in a sound-protected enclosing. Lights were illuminated over the chamber and over two nosepoke ports at the start of the behavior. Nosepoke ports were placed on the chamber wall on either side. When subjects poked an active nosepoke port, optical stimulation was activated and stimulation was delivered as described in section (4.1.2) but only for 2 seconds, without repeated activation from a single press. Inactive nosepokes did not generate any light responses.
2.3 Results

2.3.1 Preparation Validation

We began by validating the viral expression, lens-implant targeting and optogenetic efficacy of our preparation for the behavioral tasks that followed. Viral injection showed robust expression of both ChR2-YFP (Fig 3A) and NpHR-YFP (Fig 4A) in both core and shell of the NAc with expression restricted to Cholinergic interneurons (Fig 3C and 4C). Optical Lens targeting showed lens termination in the medial regions of the NAc with sphere of light penetration poised to optimally illuminate both shell and core substructures (Fig 3A and 4A). Ex vivo preparations were then employed to validate the efficacy of our optogenetic stimulation paradigms and robust responses were recorded in these preparations with high temporal fidelity (Fig 3D and 4D).
Figure 3 - A. Cre-dependent ChR2-YFP (or YFP-only) virus was injected bilaterally into the NAc of CHAT::IRES-Cre mice and fibers were implanted above the injection sites. B. Top: ChR2 expression in cholinergic interneurons. Scale bar: 300um. Bottom: summary of fiber tip locations. C. Co-localization between ChR2-YFP and ChAT immunohistochemistry. Scale bar: 20um. D. Whole cell recordings to confirm the functionality of ChR2 in slices prepared 4-6 weeks after infusion. Stimulation paradigm: 445 nm light, 5 mW/mm2, 15 Hz, pulse duration: 5 ms, 2s of stimulation paired to a 2 second laser off interval.
2.3.2 Cholinergic Activation Enhances Cocaine CPP Extinction Learning

We examined how activation of cholinergic interneurons modulates extinction learning of a cocaine-associated context. We found that relative to their YFP controls, subjects receiving optogenetic cholinergic stimulation during the first extinction trial (Test 1) spent less time spent on the cocaine-conditioned chamber across all three extinction test days (Fig 5A and B; F(2,34)=10.31 p=0.0003 for test day and F(1,17)=5.141 p=0.0367 for group, repeated measures ANOVA). Intriguingly, the reduction in preference between the cholinergic stimulation group and their YFP controls persisted on subsequent extinctions (tests 2 and 3) even

Figure 4 - A. Cre-dependent NpHR-YFP (or YFP-only) virus was injected bilaterally into the NAc of ChAT::IRES-Cre mice and fibers were implanted above the injection sites. B. Top: NpHR expression in cholinergic interneurons. Scale bar: 300um. Bottom: summary of fiber tip locations. C. Co-localization between NpHR-YFP and ChAT immunohistochemistry. Scale bar: 20um. D. Whole cell recordings to confirm the functionality of NpHR in slices prepared 4-6 weeks after infusion. Stimulation paradigm: constant illumination at 590 nm light.
though cholinergic activation only occurred only on test 1. This persistence implies that cholinergic activation enhances the learning of an extinction context as opposed to temporarily altering either acute craving or expression of memory.

2.3.3 Cholinergic Inhibition Impedes Cocaine CPP Extinction Learning

Given our finding that cholinergic activation enhances extinction learning, we next sought to examine whether inhibition of these neurons might exhibit the opposite effect and impede extinction learning. We found that optogenetically inhibiting cholinergic interneurons indeed disrupted extinction learning relative to YFP controls (Fig 6A and B, $F(2,37) = 15.23, p < 0.0001$ for test day and $F(1,18) = 5.63, p = 0.029$ for group, repeated-measures ANOVA). As with activation of
cholinergic interneurons, the effects of cholinergic inhibition persisted over subsequent extinctions tests, further suggesting that these effects come from learning. Collectively, these experiments demonstrate that cholinergic activity conveys bidirectional control over learning for a cocaine-extinction context.

Figure – 6. Heatmaps representing time spent in the saline and cocaine chamber on “Test 1 of an example mouse expressing either NpHR-YFP virus (top) or YFP-only virus (bottom). B. Average cocaine chamber preference during the pre-test and the three test days for mice undergoing cocaine CPP (F(1,17)=5.6 p=0.02 for group, repeated measures ANOVA. All error bars are SEM.

2.3.4 Controlling for Non-Specific Effects

Given that the cholinergic interneurons of the NAc exhibit bidirectional control in cocaine-context extinction, we would like to account for other kinds of cognitive or behavioral processes aside from learning, that might confound this finding. One such possibility is that the effects we demonstrated in cocaine CPP extinction derive from non-specific changes in anxiety or locomotion from
cholinergic manipulation, rather than learning as such. This seems unlikely given that we witness durability in behavioral changes in extinction trials after our optogenetic intervention. Nevertheless, previous research has characterized some degree of influence of acetylcholine transmission on anxiety (Labarca, 2001) and striatal cholinergic transmission has been shown to alter locomotion (Drenam, 2010). We therefore set out to determine whether cholinergic Interneuron transmission in the NAc influences anxiety and locomotion and employed the open field test (OFT) and elevated plus maze (EPM) as behavioral assays of these behaviors.
We determined that the behavioral effects in Cocaine CPP extinction from cholinergic Interneuron stimulation were unlikely to be caused by nonspecific effects in either velocity or anxiety-like behaviors. For both activation and inhibition
interventions, assays such as the elevated plus maze and the open field test showed no evidence of anxiogenesis as measured by open arm time (Fig 7A and Fig 8B) and center time (Fig 7D and Fig 8A) respectively. In addition, relative to controls, optical manipulation did not alter velocity in the case of either the open field test (Fig 7B and Fig 8C) or the first cocaine CPP extinction test (Fig 7B and 8D).

Figure – 8 Various behavioral measures were not significantly altered by cholinergic interneuron inhibition in experimental animals compared to littermate controls. A. Mean open arm time in an elevated plus maze task. Open arm time was calculated for 3 epochs, each of 3 minute duration, with optogenetic activation only during the second epoch. (F(1,10)=0.072, p=0.79 for group, repeated measures ANOVA.) B. Mean velocity during the open field test (two tailed t-test, p = .64). C. Velocity of mice during extinction test 1 during the cocaine CPP (two tailed t-test, p = .32). Mean time spent in the center area of a 5 minute open field test (optogenetic stimulation throughout the test) (two tailed t-test, p = .54). All error bars are SEM.
2.3.5 Cholinergic Stimulation has no Effect on Intra Cranial Self Stimulation or Real Time Conditioned Place Preference

Given that non-specific effects could not account for our findings, it is likely that the effects we demonstrated in cocaine CPP extinction derive from learning. An important question to resolve and one of our chief motivations, is to determine exactly how cholinergic interneuron stimulation affects learning in these cases. One possibility is that cholinergic interneuron stimulation could alter cocaine-context extinction learning because these neurons directly cause reinforcement, like dopamine. However, cholinergic interneurons could also enhance cocaine-context extinction by modulating ongoing reinforcement, serving as kind of “gain function”. We next sought to determine whether cholinergic activation was sufficient, by itself, to reinforce behavior in naïve animals by activating cholinergic interneurons both for intracranial self stimulation (ICSS), and during a real-time (RT) CPP.

Optical activation of cholinergic cells did not alter the balance of time spent between the two chambers which suggests that cholinergic activation by itself does not carry a reinforcement signal (Fig 9B) and cholinergic activation also failed to reinforce intracranial self-stimulation (ICSS) (Fig 9A) Similarly, optical inactivation failed to alter a RT CPP (data not shown (see Lee et al., 2016 for details)) Together, these findings argue against the hypothesis that cholinergic interneurons instruct new learning.
2.4 Summary of Findings

The aim of this chapter was to determine the effects of cholinergic interneuron activity on cocaine CPP extinction and better characterize the relationship between cholinergic activity and reinforcement. We determined that manipulating cholinergic interneuron activity bidirectionally modulates extinction learning for a cocaine CPP. We next illustrate that cholinergic interneuron activity, by itself, is insufficient to induce reinforcement of behavior, suggesting that cholinergic interneurons, unlike dopamine neurons, do not instruct reinforcement...
by themselves. Thus our activation, by itself is unlikely to account for changes in extinction by inducing new reinforcement and suggests that cholinergic interneurons are modulating ongoing enforcement instead. In subsequent behavioral paradigms, and analyses, we concluded that such effects could not be explained due to non-specific effects of our manipulation on locomotion or anxiety.
3. Cholinergic Interneuron Activation Has no Effect on Extinction of Fear and Food Context Associations

3.1 Introduction

Given that cholinergic interneurons stimulation affects bidirectional control over the extinction of a cocaine CPP, an important question to address is whether cholinergic interneuron stimulation controls context extinction for other types of context learning. We set out in this chapter to expand our previous finding and examine whether cholinergic activation could influence other domains of context extinction for both food-context associations and fear-context associations.

While it is possible that cholinergic interneuron stimulation influences food reward-context extinction learning as it does cocaine-context extinction, cocaine induces reinforcement in the NAc in ways which are not comparable to natural reinforcers such as sucrose or food (Carelli et al., 2000; Olsen, 2011; Opris et al., 2009). Unlike these natural rewards, cocaine directly exhibits powerful and lasting pharmacological effects on plasticity and learning and pushes neuromodulator transmission beyond a homeostatic dynamic range (Chen et al., 2008; Mameli et al., 2009). However, given the role of NAc acetylcholine in signaling satiety, a role for cholinergic neurons influencing food reward-context association comprises a plausible hypothesis. In this chapter, we examine the role of cholinergic
Interneurons during extinction learning for food context association using a food conditioned place preference paradigm.

Fear context learning comprises another important category of context association and though cholinergic transmission plays an intriguing role in facilitating fear-context learning in the BLA (Jiang et al., 2016) and Hippocampus (Hersman et al., 2017), as well as in the NAc. Two pieces of evidence in particular support the hypothesis that cholinergic interneurons in the NAc can contribute to fear context learning. Recent work shows that GABA projecting VTA axons selectively control cholinergic interneurons of the NAC and that activating these projections “paused” cholinergic activity and enhanced discrimination of fear stimuli (Brown et al., 2012). Additionally, inhibition of cholinergic interneurons during fear context acquisition enhanced the expression of freezing when animals were exposed to the conditioned context the next day (Witten et al., 2010). These findings suggest an important role for cholinergic transmission in the NAc for both context and cue conditioned fear learning. This evidence, coupled with our own evidence that cholinergic interneurons could impact cocaine-context extinction lead us to hypothesize that these neurons might also play a role fear context extinction. We set out to test this hypothesis by stimulating cholinergic interneurons while animals underwent context extinction after a fear conditioning paradigm.
3.2 Methods

We prepared optogenetic cohorts to provision precise viral targeting of cholinergic interneurons in the NAc with ChR2 and implanted optical fibers as described in the previous chapter (see section 2.2.1 for details). All surgical procedures, histological and immunohistochemical methods and all stimulation parameters for activation of ChR2 were duplicated from previous experiments in Chapter 2 (see section 2.2.2).

3.2.1 Food CPP

4-6 weeks after surgery, a behavioral cohort (19 CHAT::IRES-Cre mice (ChR2, n=9, YFP, n=10)) was conditioned on a food CPP. We placed subjects in the same two chamber arena employed in chapter 2 (see section 2.2.6) separated by a central portal. On the first day, each mouse was placed in the center of the chamber while connected to patch cables that were not emitting light and were permitted to explore the chamber freely, giving us an initial measure of native preference (pre-test). Day 2 through 6 consisted of conditioning, wherein each mouse was confined to one of the side chambers for 30 minutes in the morning and then again, to the opposite chamber in the afternoon for an additional 30 minutes. One chamber had Kellog’s fruit loops spread evenly over the entire side, while the other was empty. Subjects underwent 3 extinction tests to measure and extinguish preference for the cocaine associated side. Day 7 consisted of the 1st extinction trial (Test 1) where mice received blue light stimulation during the 15 minutes in which they had access to both chambers in the two chamber apparatus.
On day 8 and 16 (Test 2 and 3), mice were again placed in the center chamber and allowed to freely explore both chambers for 15 min without stimulation (Test 2 and 3). Of note, all subjects undergoing food CPP were placed under a strict *food restriction regimen* to consume 85% of their standard food intake (this excludes 30-minute fruit loops access). 24 hours before the first food conditioning, subject began the food restriction protocol. The weight of all subjects was recorded on every other day and subjects lost an average of 10% of their starting weight by the last extinction trial (Fig 10).

![Fig 10. Subjects were trained on a food conditioned place preference task with CHOLINERGIC interneuron stimulation delivered throughout "Test 1", the first post-conditioning test, but not subsequent post-conditioning tests.](image)

### 3.2.2 Fear Conditioning

Fear conditioning was performed in a sound-protected acoustic chamber (Coulbourn instruments, PA, USA) protecting an 18 x 18 x 30 cm cage wired to a shock generating device, coupled to a grid floor. In order to induce fear conditioning, subjects were put into the fear conditioning chamber. At two minutes, the first foot shock (0.5 mA, 2 s) occurred and a second shock was delivered again, two minutes after the initial shock. After the delivery of the second shock, subjects were left in the fear conditioning chamber for 30 more seconds before being
returned to their home cages. Extinction tests were performed over the following 2 days, wherein subjects were returned to the fear-conditioned context without shock (Fig 11). During the test, freezing was measured for 5 min and optogenetic stimulation was delivered for the entire of the first of the two extinction tests. Freezing was measured in each trial by a blind and experienced experimenter.

Figure 11 – Subjects underwent fear conditioning and received optogenetic activation of CHOLINERGIC interneurons throughout the “Test 1”, the first post-conditioning extinction test, but not “Test 2”, the second post-conditioning test.

3.3 Results

To our surprise, our findings that activation of cholinergic interneurons influences extinction learning for cocaine-context association did not extend to either natural reward or fear context association (Fig 12). For food CPP, activation of cholinergic interneurons during the first extinction test evidenced no effect on food chamber extinction neither in test 1, where subjects received stimulation or in subsequent tests. Similarly, for extinction of a contextual fear memory, activation of cholinergic interneurons showed no effect of freezing for tests on either of the two extinction days.
3.4 Discussion

There are several reasons why our paradigm of cholinergic interneuron stimulation may have influenced one type of context-extinction learning over others. To begin with, we are hardly the first group to show that specific NAc manipulations engender differential results on learning for different kinds of reinforcers. Sucrose and cocaine recruit differential pathways for plasticity (Hong, 2006) and additionally, numerous studies show differential region-specific influence and activity for drug vs. food based rewards (Counotte et al., 2014). Given these stipulations, a likely explanation for the specificity of our effects for cocaine-context extinction learning likely pertain to the influence of our
interventions on structural and plastic underpinnings more specific to cocaine reward.

Furthermore, that cocaine, but not fear learning, was influenced by cholinergic activation, should not necessarily come as a surprise, since earlier research demonstrates entirely opposite influences of NAc cholinergic inhibition on fear and cocaine acquisition learning respectively (Witten et al., 2010). This suggests that cholinergic interneurons have separable roles for supporting fear vs cocaine context learning. That our stimulation paradigm supports learning in one condition but not the other can be understood plainly to suggest that the operations that these neurons perform to support learning are likely to be highly differentiable in these cases. For example, cocaine has been shown to elevate cholinergic interneuron activity over long time periods, gradually increasing after administration. By contrast, the activity of cholinergic interneurons to salient events such as shocks tends to be more punctuated. It is likely therefore that extinction for these reinforcers is supported by differential activity. Future research should examine neural correlates in these cases to better understand such distinctions for differential reinforcers as they pertain to extinction learning.
4. Linking Cholinergic Interneuron Activation to Plasticity

4.1 Introduction

4.1.1 How do Cholinergic Interneuron-Driven Changes in Context Extinction Learning Relate to Plasticity?

In this chapter we penetrate further into why cholinergic activation alters cocaine CPP, but not food or fear context-extinctions and we theorize that changes in cholinergic driven synaptic plasticity likely distinguish these outcomes. If this is true, it would suggest that cholinergic activation might alter plasticity in the case of a cocaine, but not a food context extinction. That synaptic plasticity could underlie acetylcholine’s effects on learning stands to reason given its well characterized role as a neuromodulator: Acetylcholine is known to alter synaptic plasticity throughout the brain, (Higley et al., 2009; Ji et al., 2001; Power and Sah, 2008; Wang et al., 2006). Yet how cholinergic modulation at specific synaptic pathways impacts behavior and learning is not clear. We have already demonstrated that cholinergic neurons modulate, rather than cause ongoing learning. Could it also be that cholinergic interneurons modulate, rather than directly cause, ongoing changes in plasticity? In this Chapter we systematically analyze the relationship
between cholinergic activation, changes in behavior and changes in synaptic plasticity.

4.1.2 Cocaine-Context Learning Causes Changes in NAc Synaptic Plasticity

We reasoned that cholinergic interneurons might impact extinction learning by modulating ongoing synaptic plasticity in the NAc and theorized that such changes must inherently occur during cocaine context extinction. Many groups show staged changes in NAc synaptic plasticity natively underlying distinct phases of drug-adaptive behaviors (Everrit 2005; Kalivas and Volkow, 2005; Kalivas and O’Brien, 2008). Yet to our knowledge, none have convincingly or as thoroughly characterized such changes in NAc plasticity to support extinction learning in cocaine context-extinction. There is nonetheless, reason to suspect that such plasticity does in fact underlie cocaine-context extinction learning, with reports of increases in NAc of the intermediate early gene c-fos for mice undergoing cocaine context extinction vs. cocaine adapted controls who underwent sham-context extinction (Orsini et al., 2013). Characterizing the plasticity that supports drug-context extinction thus comprises an important gap to fill in systems neuroscience – Our motivation in this chapter is to contribute to a more comprehensive characterization of how staged plasticity underlies drug adapted behaviors in the case of drug-context extinction learning.
Beyond its relevance to systems neuroscience research though, it is essential to better characterize synaptic mechanisms that support drug-context-extinction learning for clinical purposes as well. Such findings could merit relevance for clinical populations and provide insight for potential advances for translational research. This is particularly true in the case of drug context extinction learning, because contextual cues illicit drug seeking responses and give rise to relapse.

We thus begin this chapter with an intriguing experiment: do groups which undergo repeated extinction trials, compared to idle, cocaine-adapted controls, differentially yield notable changes in NAc synaptic plasticity? If so, what kind of plasticity, if any, occurs in such cases? To answer these questions and for the reasons we have outlined above, we deploy our cocaine CPP extinction task, and ex-vivo physiology, to illuminate underlying correlates of neural plasticity in NAc MSNs from cocaine context extinction.

4.1.3 Cholinergic Interneuron-Driven Changes in Plasticity Distinguish Outcomes between Cocaine and Food Context Extinction Learning.

Since cholinergic activation imposes distinct outcomes on Food and Cocaine context extinction, we next chose to examine plasticity in the NAc in both cases. Do differences in behavioral outcomes reflect distinctions in underlying changes in cholinergic interneuron-driven plasticity? We theorized that cholinergic
interneuron activation selectively facilitates glutamatergic plasticity on MSNs in the case cocaine, but not food conditioned context extinction. In support of this theory, existing literature demonstrates that specific kinds of NAc plasticity selectively cause underlying cocaine-adapted, but not natural reward related behaviors (Bock et al., 2013; Counotte et al., 2013; Smith et al., 2013; Pascoli et al. 2012). In fact, in many cases, specific interventions can cause alter glutamatergic synapses or input in the NAc to alter cocaine adaptive behaviors, while leaving natural reward related behaviors intact (Bock et al., 2013; Ma et al., 2014; Lee et al., 2013; Pascoli et al., 2014). In this chapter, an important motivation for us is to resolve the relationship between when cholinergic activation facilitates changes in behavior and when it induces changes in plasticity.

To this end, we again perform food and cocaine conditioned place preference, and, in both cases, we sacrifice subjects after the first extinction learning task to examine differential effects of cholinergic interneuron-driven plasticity on learning. We employ ex-vivo physiological recordings on MSNs in prepared sections of the NAc to examine subsequent changes in glutamatergic synaptic plasticity. We show evidence to support the hypothesis that cholinergic activation during extinction learning facilitates presynaptic glutamatergic plasticity. We show that changes in behavior in cholinergic mediated cocaine context extinction predict changes in miniature excitatory post-synaptic currents (mEPSCs), a measure of strength for excitatory synapses, onto MSNs. We show that these changes in mEPSC strength are not being induced by cholinergic
activation by itself however. Activation of cholinergic interneurons in slice fails to induce changes in plasticity on these measures.

4.1.4 Cholinergic-Driven Changes in Plasticity are Context and Experience-Dependent

In conjunction with cases in which they modulate learning, could cholinergic interneurons also serve to modulate, rather than instruct ongoing plasticity? To experimentally determine the answer to this question, we will stimulate cholinergic interneurons a cocaine-CPP cohort, but this time, we will vary the context in which the stimulation is delivered for extinction. Will we witness cholinergic-interneuron driven changes in plasticity and extinction learning when cocaine adept animals are stimulated in their home-cage rather than in a learning context? One hypothesis is that cholinergic activation generates glutamatergic synaptic plasticity irrespective of experience and context. The alternative hypothesis is that cholinergic activation influences the extent of plasticity when new learning in cocaine-context extinction is occurring. In this section, we set out to distinguish experimentally between these two hypotheses. We theorized that cholinergic interneuron stimulation must be coupled to ongoing learning, within a learning context to modulate ongoing plasticity and behavior. Findings here reach to the heart of our understanding of this important neuromodulatory population.
4.2 Methods

We prepared optogenetic cohorts to provision precise genetic-population targeting for all behavioral cohorts in this chapter. All surgical procedures, histological and immunohistochemical methods and all stimulation parameters were duplicated from previous experiments (see Chapter 2). In this case, both control and stimulation cohorts animals were injected with Cre-dependent ChR2 and comparisons were made between stimulated subjects vs. no stimulated controls.

4.2.1 Behavioral Assays and Stimulation Paradigms

We again employed the cocaine CPP paradigm from chapter 2 (see section 2.2.6) and Food CPP paradigm from chapter 3 (see section 3.2.1) in this chapter and concomitant stimulation paradigms (See section 2.2.3). We detail deviations in this protocol to provision for ex-vivo physiology. Animals were sacrificed directly after specific extinction tests in these cases (See section 4.2.2 and 4.2.3 for details).

4.2.2 Repeated Extinctions

We trained animals again on a cocaine conditioned place preference, but this time, without any cholinergic stimulation for either an extinction group, or their littermate controls (Fig 13). The extinction group underwent repeated extinctions tests on 4 occasions over subsequent days after training while littermate controls did not receive any extinction tests. At the end of the 4 extinction tests, or the end
of the 4th post-conditioning day for littermate controls, animals were sacrificed and NAc sections were prepared for ex-vivo physiology.

Figure 13 – Cocaine CPP paradigm followed by 4 extinction sessions in the extinction group and no extinction in the control group. Both groups were sacrificed at the same time point, immediately after the final extinction test.

4.2.3 Cholinergic Activation Vs. No Activation in a Cocaine Context

Extinction

We compare the effect of cholinergic stimulation during a cocaine context extinction task, to littermate controls that undergo the first extinction trial, but without stimulation (Fig 14). The cocaine CPP behavioral paradigm is run again as it was in chapter 2 (see section 2.2.6) but this time, animals are sacrificed immediately after the first extinction trial and sections are prepared for ex-vivo physiology.
4.2.4 CHOLINERGIC Activation Vs. No Activation in Food Context Extinction

We compare the effect of cholinergic stimulation during a food context extinction task, to littermate controls that also undergo the first extinction trial, but without stimulation (Fig 15). The food CPP behavioral paradigm is run again as it was in chapter 3 (see section 3.2.1) but this time, animals are sacrificed immediately after the first extinction trial and sections are prepared for ex-vivo physiology.

Figure 15 – Subjects underwent Food CPP and received optogenetic activation of cholinergic interneurons or sham activation throughout the first conditioning extinction test. Subjects were sacrificed immediately after the test and sections were prepared for NAc ex-vivo electrophysiology.

4.2.5 Cholinergic Activation During Vs. Before Cocaine Context Extinction

We compare the effect of cholinergic stimulation during a cocaine context extinction task, to littermate controls that also undergo the first extinction trial, but receive stimulation beforehand for 15 minutes in their homecage (Fig 16). The
cocaine CPP behavioral paradigm is run again as it was in chapter 2 (see section 2.2.6) except animals are sacrificed immediately after the first extinction trial and sections are prepared for ex-vivo physiology.

4.2.6 Ex-Vivo Physiology – mEPSC recordings.

Slice perpetrations were made as detailed in chapter 3 (section 3.1.4) with modifications to accommodate miniature EPSCs (mEPSCs) recordings. mEPSCs were recorded in the presence of TTX (1 μM), d-AP5 (50 μM) and Picrotoxin (100 μM) in the recording ACSF solution and analyzed with MiniAnalysis (Synaptosoft) using detection threshold of >7 pA and rise time <3 ms and the results were visually verified. For each cell a stretch of 300 mEPSCs were analyzed, with data collected approximately 10-15 minutes after patching onto each cell.
4.3 Results

4.3.1 Repeated Cocaine-Context Extinctions Reduce the Frequency of mEPSCs onto MSNs

To determine whether changes in plasticity natively support changes in behavior in cocaine-context extinction, we compared measures of plasticity for a group undergoing repeated cocaine context extinction versus a idle control. Repeated extinctions served to reduce preference for the cocaine associated chamber (Fig 17A). Consistent with our hypothesis, the repeated extinction group exhibited a reduction in the frequency of mEPSCs onto NAc MSNs (Fig 17B-Right) relative to a control group. Measurements for both groups were taken immediately after the fourth extinction test, or in an idle control condition, in separate groups of mice that were sacrificed at that time point. This supports our hypothesis that extinction natively modulates glutamatergic plasticity onto MSNs in the NAc. Notably, extinction did not alter mEPSC amplitude between the two groups (Fig 17B-Left). This suggests that the site of the plasticity is presynaptic. This result is permissive of our hypothesis that cholinergic activation modulates the extent of ongoing plasticity because it confirms that plasticity occurs natively from repeated exposure to extinction for a cocaine-context.
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**Cholinergic Activation Reduces the Frequency of mEPSCs onto MSNs in Cocaine Context Extinction**

We next sought to determine whether cholinergic activation altered plasticity in the case of cocaine context extinction learning. We compared mEPSC frequency and amplitude for groups who had stimulation paired to extinction vs no-stimulation controls. Consistent with our earlier results, cholinergic activation delivered during the extinction test significantly reduced chamber preference compared to no-activation controls, suggesting that cholinergic activation facilitated extinction learning (Fig 18A). We then compared mEPSCs onto MSNs in prepared NAc slices from the 2 groups that had undergone extinction (Fig 18B). Measurements for both groups were taken immediately after the first extinction test.

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**Figure 17** — Cocaine chamber preference during the baseline and four extinction tests for mice undergoing the CPP described in section 4.2.2. **A.** Left: Cumulative probability of interevent intervals for mEPSCs in extinction group and control. Decreased mEPSC frequency in extinction group. (p=0.0135 for group in a linear, mixed effects regression. Left inset: Median frequency of mEPSCs, Extinction group. Right: Cumulative probability of the amplitude mEPSCs in extinction group and for control. No change in mEPSC amplitude in both groups (p=0.697 for group in a linear, mixed effects regression. Right inset: Median amplitude of mEPSCs. All error bars are SEM.

**4.3.2 Cholinergic Activation Reduces the Frequency of mEPSCs onto MSNs in Cocaine Context Extinction**
in separate groups of mice that were sacrificed at that time point. We found a sharp decrease in mEPSC frequency onto MSNs only in subjects that had cholinergic interneuron activation delivered during the extinction (Fig 18C - Left). By contrast, no significant changes in mEPSC amplitude were detected between the two groups (Fig 18C – Right). This supports our hypothesis that when cholinergic interneurons impact extinction learning, they do so by modulating synaptic glutamatergic plasticity onto MSNs in the NAc. We again showed evidence that the site of this glutamatergic plasticity was presynaptic, since we witnessed no change in amplitude between groups. This time however, our hypothesis was further confirmed by our finding that this same intervention greatly enhanced the paired pulse ratio (PPR) on MSNs, an assay of presynaptic plasticity (Data not shown, (see Lee et al., 2016 for details)).
Figure 18 - A. Prior to ex vivo physiology, mice exhibited less preference for the cocaine paired chamber if cholinergic interneurons were activated during the test (relative to control mice which did not receive cholinergic interneuron activation but otherwise underwent the same behavior) (F(1,15)=32.1 p<0.0001 for group and F(1, 15)=8.396 p=0.011 for test, two-way ANOVA with group and test as factors.). B. Sample voltage clamp recordings of mEPSCs from an MSN from one mouse that received cholinergic stimulation and from a control mouse. C. Left: Cumulative probability of interevent intervals for mEPSCs in mice that received cholinergic activation during the test and for control mice. Decreased mEPSC frequency in mice that received stimulation. (p<0.001 for group in a linear, mixed effects regression). Left inset: Median frequency of mEPSCs. Right: Cumulative probability of the amplitude mEPSCs in mice that received cholinergic activation and for control mice. No change in mEPSC amplitude in mice that received stimulation (p=0.11 for group in a linear, mixed effects regression). Right inset: Median amplitude of mEPSCs, cholinergic activation group. All error bars are SEM.
4.3.3 Cholinergic Activation in NAc Sections Fails to Induce Glutamatergic Plasticity on Its Own

One possible explanation for these cholinergic driven changes in mEPSC frequency from cocaine context-extinction, is that cholinergic activation natively changes mEPSC frequency, irrespective of context and learning. To isolate the effects of cholinergic activation on NAc MSNs, we delivered an identical stimulation paradigm in NAc sections of naïve animals and recorded subsequent changes in mEPSC frequency over time relative to a 5 minute pre-stim baseline. We found that cholinergic activation did not alter mEPSC frequency by itself (Fig 19), suggesting that activation by itself was unlikely to account for cholinergic driven changes in mEPSC frequency from cocaine context-extinction.

![Figure 19](image)

Figure 19 - The amplitude of evoked EPSCs in MSNs was not altered by 15 minutes of cholinergic activation with ChR2 (5mW/mm2, 445nm, 15 hz, 5ms pulse duration, 2s light on interleaved with 2s light off for 15min). For 5 minutes before the stimulation and for 30 minutes afterwards, every 15s, electrical stimulation (0.1ms duration) was delivered through a stimulating electrode while EPSCs were measured in an MSN. Each dot represents the average EPSC amplitude during each minute, relative to the average baseline EPSC for the 5 minutes before optical stimulation (p=0.1268, two tailed t-test). All error bars are SEM.
4.3.4 Cholinergic Interneuron-Activation Fails to Reduce the Frequency of mEPSCs onto MSNs during a Food Context Extinction

We next sought to determine whether cholinergic activation could influence plasticity in a Food CPP compared to a no-stimulation control. Measurements of mEPSC frequency and amplitude for both groups were again taken immediately after the first extinction test in separate groups of mice that were sacrificed at that time point. We found that activation of cholinergic interneurons during a food conditioned place preference, as previously reported, failed to alter extinction learning for a food CPP (Fig 20A). Furthermore, in accordance with its lack of effect on behavior, we found that there was no cholinergic-interneuron-mediated change in the frequency or amplitude of mEPSCs between groups (Fig 20B). This further supports our hypothesis that underlying changes in behavior from cholinergic activation are supported by plasticity. It suggests that in the cases when cholinergic stimulation fails to alter behavior, it also fails to modulate plasticity.
Finally, we sought to examine whether or not cholinergic interneuron driven changes in plasticity were dependent upon the experience and context of the animal receiving the stimulation. To accomplish this, we compared cholinergic interneuron-mediated plasticity in animals trained on a cocaine CPP as before and compared the effect of cholinergic interneuron activation during the first extinction test, versus activation immediately before the extinction test in the home cage. As with our previous results, cholinergic activation led to reduced cocaine chamber preference when stimulation was delivered throughout the first extinction test.

4.3.5 Cholinergic Interneuron-Driven Changes in Behavior and Plasticity are Context-Dependent

Figure 20 - A. Prior to ex vivo physiology, Mice in which cholinergic interneurons were activated during the first extinction session of a food CPP exhibited no difference in chamber preference relative to non-stimulated control mice (F(1,9)=0.031 p=0.863 for group and F(1,9)=32.19 p=0.0003 for test, two-way ANOVA. B. cholinergic Left: cumulative probability of interevent intervals for mEPSCs in food-conditioned mice that received cholinergic stimulation versus control mice. No significant difference in mEPSC frequency across groups (p = 0.964 for group in a linear regression). Left inset: median frequency of mEPSCs. Right: cumulative probability of the amplitude of mEPSCs in mice that received stimulation versus the control condition. No significant difference in mEPSC amplitude across groups. (p = 0.57 for group in a linear regression. Right inset: median amplitude of mEPSCs.
However, the group receiving stimulation before the extinction test evidenced robust chamber seeking behavior during the first extinction test by comparison (Fig 21A). Consistent with this result, the group that received activation of cholinergic interneurons during extinction learning also displayed a reduction of mEPSCs relative to the group that had received stimulation beforehand (Fig 21B-Left). No changes in mEPSC amplitude significantly emerged between the two groups (Fig 21B-Right). This finding suggests that cholinergic activation generates glutamatergic synaptic plasticity in a way that is context and experience dependent because activation was dependent on experience in a cocaine extinction context to alter plasticity and behavior. Furthermore, since cholinergic neurons do not specify plasticity on their own, it further suggests the hypothesis that cholinergic activation instead influences the extent of plasticity when new learning in cocaine-context extinction is occurring.
Figure 21 – A. Mice in which cholinergic interneurons were activated during the extinction test showed less preference for the cocaine-paired chamber before whole cell recordings (relative to mice which received the cholinergic interneuron stimulation in the home cage but otherwise had the same behavior experience) (F(1,16)=4.703 p=0.0455 for group and F(1,16)=14.24 p=0.0017 for test, two-way ANOVA with group and test as factors; p=0.99 for Pre-test, p=0.013 for Test, Sidak’s post-hoc test) B. Left: Cumulative probability of interevent intervals for mEPSCs in mice that received cholinergic activation during the test and mice that received cholinergic activation in the home cage. Decreased mEPSC frequency in mice that received stimulation during the test. Right: Cumulative probability of the amplitude of mEPSCs in mice that received cholinergic activation during either the test or the home cage (p=0.393).
5. Overview

5.1 Summary of Results

With high temporal and genetic specificity, we investigated the role of cholinergic interneurons of the NAc in a mouse model system using extinction of a cocaine conditioned place preference as a behavioral paradigm. The findings in this thesis leads us to four principle conclusions:

1) **Cholinergic neurons implement bidirectional control over extinction learning for a cocaine CPP.** This was determined by demonstrating that the optogenetic activation and inactivation of cholinergic interneurons of the NAC reduced and enhanced the expression of cocaine-chamber preference respectively during an extinction test. We established that these alterations were in fact due to learning, because they endured in tests without stimulation.

2) **Cholinergic interneurons of the NAc do not instruct learning or plasticity like DA neurons do.** We established this by showing that stimulation of cholinergic interneurons of the NAc fails to condition either conditioned place preference or aversion and could not drive plasticity by itself.

3) **During cocaine-context extinction learning cholinergic activation alters presynaptic plasticity on MSNs.** When we employed ex-vivo
physiology it showed a reduction in the frequency of miniature postsynaptic currents in cases when cholinergic activation was paired to cocaine, but not food CPP extinction.

4) **Cholinergic interneurons modulate ongoing learning and plasticity and they do so in a context and experience dependent fashion.** We demonstrate that repeated cocaine CPP extinctions natively generate the type of plasticity that cholinergic activation enhances. We then demonstrate context dependence by showing that stimulation outside of an extinction learning context, for cocaine conditioned subjects, fails to influence subsequent learning and plasticity.

### 5.2 Discussion

#### 5.2.1 Cholinergic Manipulation Effects on Behavior

To summarize, our findings suggest a role for cholinergic interneurons in the NAc in which they modulate cocaine, but not food or fear-context extinction learning. Cholinergic interneuron activation and inhibition modulate the extinction of a cocaine-context association bidirectionally yet do not generate reinforcement or learning by themselves. These manipulations could not reinforce chamber aversion or preference for a real-time CPP, wherein a subject can freely ambulate between sides in a CPP chamber and optogenetic stimulation is paired to one side.
5.2.2 Cholinergic Manipulation Effects on Plasticity

In a similar fashion, cholinergic interneuron activation modulates glutamatergic synaptic plasticity onto MSNs but only when coupled to learning during extinction of a cocaine CPP. Of note, in conditions where cholinergic interneurons do not influence learning or behavior, they exhibit no effects on plasticity. We determined that cholinergic interneuron activation during cocaine-context extinction specifically modulates measures of presynaptic glutamatergic plasticity: reduced mEPSC frequency and enhanced paired pulse ration of MSNs. However, plasticity from cholinergic activation seemed to rely on co-occurrence with learning during cocaine CPP extinction. When animals underwent food CPP, cholinergic activation failed to alter learning, and we saw no evidence of this plasticity. Furthermore, when animals who had undergone cocaine CPP received the same activation pattern in mice in their home cage, rather than in an extinction context we saw no evidence of changes in behavior or plasticity. Additionally, we found that slice activation of our stimulation paradigm in cholinergic cells failed to alter plasticity, as did administration of this stimulation to animals conditioned on only saline in a CPP (Data not shown, See Lee et al., 2016 for details).

5.2.3 A Model for When Cholinergic Manipulation Alters Behavior and Plasticity

Taken together, we demonstrate conditions under which cholinergic interneuron activation alters behavior and conditions under which it alters plasticity.
and our work establishes a convincing relationship and tight correspondence between the behavioral and plasticity effects that cholinergic interneurons mediate. We interpret these results to support a model in which cholinergic interneurons in the NAc can hasten plasticity that natively occurs to support ongoing learning but cannot generate plasticity or reinforcement on their own. We demonstrate support for this conception by showing that repeated extinction tests in cocaine CPP natively reduces the mEPSC inter-time intervals on MSNs in ways that closely mirrors changes induced by cholinergic activation during a single cocaine CPP extinction test.

### 5.2.4 Contrasting Cholinergic Interneuron Activation with the role of Dopamine in the NAc

This model of cholinergic function in the NAc, to modulate ongoing learning and plasticity, contrasts sharply with the role of midbrain dopaminergic neurons, which support reinforcement and plasticity on their own (Pascoli et al., 2015; Tsai et al., 2009; Ilango et al., 2014; Steinberg et al., 2013). While dopamine neuron activity may therefore instruct learning and determine when and where learning occurs, our findings suggest that cholinergic neurons, by contrast, influence the rate or efficiency of learning as it ensues. This distinction between how dopamine and cholinergic neurons function requires further explanation as activation of cholinergic interneurons in the NAc causes downstream terminal release of DA, thought to be mediated through DA axonal acetylcholine receptor activation.
(Cachope et al., 2012; Threlfell et al., 2012). Furthermore, in other regions, such as BLA and Hippocampus, altering cholinergic transmission is sufficient to cause changes in synaptic plasticity in vitro (Hasselmo, 2006). In contrast to our finding that cholinergic activation failed to induce changes in presynaptic activity in slice preparation, for example, photoactivation of cholinergic terminals in BLA can cause changes glutamatergic plasticity in ex-vivo preparations (Jiang et al., 2016). These findings raise considerable questions: If dopamine instructs new learning and plasticity and cholinergic activation in the NAc recruits downstream release of dopamine, why doesn’t cholinergic activation instruct new learning and plasticity? Furthermore, if other regions such as the BLA, demonstrate cholinergic photoactivation causing plasticity by itself, why doesn’t our intervention evidence this same effect? A likely explanation for both of these discrepancies is that cholinergic activation in the NAc causes the simultaneous effects on local inhibitory circuitry and acutely silences MSNs (Nelson et al., 2014; English et al., 2011; Witten et al., 2010; Faust et al., 2015). Such an effect could prevent neuromodulatory transmission from inducing reinforcement to all but the most salient glutamategic inputs, capable of overcoming an inhibitory threshold.

5.2.5 Broader Understandings of Glutamatergic Plasticity in the NAc for Drug and Reward learning; a Role for Cholinergic Interneurons

As we mentioned in the introduction, incoming glutamatergic inputs to the NAc have been shown carry information about discrete components of reward.
Recent work demonstrates that NAc projecting glutamatergic inputs encode the overlap of spatial information and social-reward for instance (Murugan et al. 2017). It is therefore no surprise that plasticity on such inputs is thought essential in supporting reward-seeking behavior. These glutamatergic inputs are thought to be modulated at the synapse by dopamine. A long-standing hypothesis holds that dopamine acts as a reinforcement signal which strengthens synaptic inputs when they predict reward. A wealth of evidence supports glutamatergic plasticity as a mechanism which supports drug-related learning (Lüscher and Malenka 2011; Stuber et al., 2010; Martin et al., 2006; Britt et al., 2012). and very convincing optogenetic experiments prove that altering synaptic strength of these inputs causes changes in maladaptive and drug-related behavioral phenotypes. (Ma et al., 2014; Lee et al., 2013; Pascoli et al., 2014). In light of the established, causal role of glutamatergic plasticity onto MSNs in drug and reward related behaviors, the role that we have demonstrated for cholinergic interneurons in modulating such plasticity is especially significant. To begin with, our results show a distinct pathway from previous optogenetic paradigms that drive long term depression on glutamatergic inputs. (Ma et al., 2014; Lee et al., 2013; Pascoli et al., 2014). In the case of previous optogenetic experiments, LTD stimulation paradigms on glutamatergic axons were employed before the behavioral tests. By contrast, the glutamatergic plasticity that cholinergic interneuron activation modulates occurs acutely, during drug related learning, while this same activation yields no effects on plasticity beforehand. A second notable contrast between our findings and previous literature on plasticity in drug-related learning is that most of this literature,
especially literature focused on withdrawal from operant self-administration, describes robust changes in post-synaptic plasticity (Conrad et al., 2008; Loweth et al., 2013; Pascoli et al., 2014; Lee et al., 2013). However, the glutamatergic plasticity from cholinergic interneuron activation which our findings describe are largely presynaptic. It is worth noting though, that such postsynaptic effects cannot be excluded from our findings. Importantly, we fail to distinguish between different MSN classes (e.g. D2 and D1 receptor type neurons) and thus, leave open the possibility that postsynaptic effects could plausibly wash out on average if they were oppositely assigned.

5.2.6 Endogenous Dynamics of Cholinergic Interneurons

Another consideration that bears discussion is that our intervention does not emulate the endogenous dynamics of cholinergic interneurons, and this poses credible challenges toward extending interpretations toward our findings about the normal functions of these neurons. Cholinergic interneurons in the striatum are thought to be tonically active (TANS) and display intriguing and reward-related dynamics in vivo, including pausing and bursting to signify reward-cues or salient events (Morris et al., 2004; Atallah et al., 2014; Joshua et al., 2008). Our 2 second optogenetic, repeated activation paradigm and continuous pausing, which we employ for virtually all of our behavioral paradigms in this research, fail to replicate the intrinsic dynamics of putative cholinergic interneurons. It is worth noting that due to learning over indiscrete time scales as well as the absence of sudden cues,
context learning, such as in CPP, comprises a significant challenge by way of linking intrinsic neural dynamics to behaviors. Yet, in spite of these obstacles, unveiling the underlying brain mechanisms that support context association and context learning itself stands as a credible and important objective for neuroscience research. To begin with, context association comprises an indispensable form of learning which recruits the orchestration of several unique nuclei and underlying mechanisms that are distinct from cue associations or instrumental learning (Torregrossa and Taylor, 2013; Fuchs et al., 2005). Arguably, though our optical paradigm did not replicate the intrinsic dynamics of putative cholinergic interneurons, it nevertheless provides novel and useful information about the role of cholinergic interneurons in these behaviors. To begin with our manipulation of cholinergic neurons was effective for bidirectional changes in behavior. Showing both necessity and sufficiency in behavior would be unlikely in the case that our intervention mirrored no underlying circuitry which natively supports the behavior itself. In addition, the synaptic plasticity on MSNs that our stimulation elicited during cocaine context extinction precisely mirrors synaptic changes that natively occur during repeated cocaine-context extinctions without any optogenetic stimulation (Fig 17). In spite of the fact that our stimulation paradigm did not replicate endogenous cholinergic interneuron dynamics, it nevertheless elicited changes in synaptic plasticity and chamber preference that mirror changes which occur under repeated extinction training. This suggests that our manipulation’s effects are interpretable in terms of their impact on downstream synaptic function and behavior.
5.2.7 Cholinergic Transmission: Distinct Significance for Cocaine and Natural Reward

Of note, our manipulation showed unique effects on cocaine context extinction, but not on food or fear context extinction, which suggests that our intervention may have significance for clinical research, irrespective of the fact that it doesn't mirror the precise intrinsic activity of cholinergic interneurons. This significance of cholinergic activation to cocaine extinction may pertain to findings which demonstrate cocaine's tremendous and persistent impact on circuit dynamics and plasticity in the NAc (Wolf and Tseng 2012; Pascoli et al., 2014; Bock et al., 2013; Stuber et al., 2010). Additionally, some evidence supports distinct and non-overlapping roles for striatal cholinergic transmission in altering food and cocaine behaviors, perhaps signified by differential dynamic activity. Earlier research comparing the effects of muscarinic blockade in the NAc show such preferential effects on cocaine over food self-administration (Mark et al., 2006) and others show selective cholinergic excitotoxic lesions in the NAc impede cocaine, but not food self-administration (Smith et al., 2004). However, more recent work shows convincingly that chemo-genetic manipulation of cholinergic cells causes bidirectional control over food consumption (Aitta-aho et al., 2017), which suggests some functional distinctions from our own activation paradigm, which had no impact on food context extinction. In combination with our own findings, it is thus clear that altering manipulation strategies for cholinergic interneurons yields differential effects on natural reward and cocaine reward behavior. Research
seeking to better characterize differences in the intrinsic cholinergic dynamics that distinguish these rewards promises to be an important avenue for future study.

5.2.8 Connecting Acetylcholine, Plasticity and Behavior

Our findings offer the first convincing evidence of a tripartite relationship between cholinergic interneurons, learning and plasticity. Each of these relationships have been described independently. Acetylcholine antagonists have been demonstrated to alter potentiation and long term depression in the dorsal striatum in slice for instance (Threlfell et al., 2012; Cachope et al., 2012). Yet the role that such modulation plays in learning and behavior has not been well characterized. Additionally, many findings show that manipulation of cholinergic transmission in the accumbens alters behavior and learning (Aitta-aho et al., 2017; Aoki et al., 2015; Brown et al., 2012; Witten et al., 2010). Yet these findings do not demonstrate a clear role for subsequent modulation of synaptic plasticity. Our findings contribute important next steps in the relationship characterized by this foundational research: They show that cholinergic interneuron activity in awake behaving animals modulates both behavior and glutamatergic plasticity onto MSNs in the NAc. Of note, the behavior and synaptic changes that a single exposure to cholinergic interneuron-driven cocaine extinction elicited mirrored the precise changes in synaptic function and behavior exhibited by exposure by multiple extinction sessions without cholinergic manipulation (Fig 17 and 18). These convergent changes in behavior and synaptic function suggest that manipulation
of cholinergic interneurons during cocaine extinction hastens the same plasticity which causes increased extinction over longer timescales. Additionally, while other studies show that cholinergic interneuron activity exhibits acute control over local GABAergic and glutamatergic activity, the synaptic plasticity our findings demonstrate differs dramatically from acute changes such as these. These acute effects are exhibited extemporaneously (English et al., 2011; Oldenburg and Ding, 2011; Witten et al., 2010; Nelson et al., 2014; Higley et al., 2011), but do not persist, whereas our stimulation drives long-lasting changes in synaptic function that persevere after stimulation durably. We found, for example, that cholinergic driven changes in synaptic plasticity from our manipulation during cocaine-context extinction, endured for 24 hours after the stimulation itself (Data not shown, See Lee et al., 2016 for details).

5.3 Future Directions

There are two critical question that our findings immediately pose upon cursory examination: Do the cholinergic driven changes in synaptic plasticity we elicit differentially segregate across distinct DA receptor subtypes in NAc MSNs? And which specific glutamatergic NAc inputs are being altered by cholinergic activation? In the first case, Dopamine receptor subtypes (D1 receptor (D1R) and D2 receptors (D2R)) of MSNs have been shown to comprise substantially distinct functional and anatomical compositions in the NAc. While both NAc D1R and D2R MSNs receive excitatory inputs from the mPFC, vHipp and BLA (Papp et al., 2012;
MacAskill et al., 2012) they are distinguished greatly in terms of their down-stream projection targets (Humphries and Prescott, 2010; Smith et al., 2013). Additionally, D1R and D2R MSNs undergo plasticity differentially in response to cocaine-learning, and recent findings show that inputs onto these populations do as well, evidencing differential plasticity during withdrawal from cocaine self-administration. Furthermore, in many of these cases, differentially altering either D1R and D2R population activity or plasticity, or input strength drastically alters drug adaptive behaviors (Bock et al., 2013; Lobo and Nestler, 2011; Pascoli et al., 2014).

Given these facts, future avenues of research must clarify if and how synapses on these distinct MSN populations are differentially affected in the case of cholinergic driven changes in plasticity and behavior. A definitive approach for providing answers to this question comprises cross-breeding CHAT::Cre animals to reporter lines that cause distinct fluorescence in either D1R or D2R MSNs. Some research supports the idea of differential responses to cholinergic neuron activation amongst these populations. Recent research has demonstrated that activating specific glutamatergic inputs onto cholinergic interneurons via thalamic stimulation in prepared brain sections alters cortical-striatal short-term plasticity for MSN synapses, in differential patterns for D1R and D2R populations. (Ding et al. 2010). Given these successes, future work should employ ex vivo physiology in NAc sections following cholinergic driven cocaine context extinction to make
determinations about changes in synaptic plasticity on D1R and D2R populations respectively.

In the second case, while we have shown cholinergic driven changes in glutamatergic plasticity onto NAc MSNs from cocaine-context extinction, it is critical that future research resolve which specific projections cholinergic interneurons modulate in this case. While it may be that all inputs are modulated selectively, there is reason to suspect that cortical inputs may be preferentially modulated over other inputs. To begin with, cortical inputs to this region have already been decisively implicated in mapping the overlap of space and reward in the case of social interaction (Murugan et al., 2017). Secondly, there is physiological evidence from the dorsal striatum, for instance, of decreases in cortical EPSCs onto MSNs elicited by cholinergic interneurons (Pakhotin and Bracci, 2007). Finally, cortical glutamatergic inputs are known contain axonal M2 and M3 receptors, which can drastically alter presynaptic glutamate efficacy (Zhou et al. 2002). Taken together, there is moderate physiological and behavioral support for the hypothesis that cortical inputs to the NAc might be responsible for effects we have shown in plasticity in this work. Future research should employ ex-vivo physiology in combination with spectrally separated optogenetic approaches to segregate cholinergic and terminal activation or chemogenetic approaches. In combination, these tools can be employed to measure changes in cortical input strength to NAc MSNs for groups that have undergone cholinergic driven extinction vs YFP control.
5.4 Conclusions

We showed that cholinergic interneuron activity in the NAc can bidirectionally alter learning during the extinction of a cocaine associated context. We determined that the mechanism by which cholinergic interneurons alters such learning is presynaptic – They induce lasting reduction in the frequency of mEPSCs onto MSNs in the NAc. Furthermore, unlike dopamine, cholinergic activity in the NAc serves to modulate, rather than cause, both learning and plasticity in such cases, as cholinergic activity is not directly reinforcing, and requires ongoing learning to exert these effects. In sum, the findings we demonstrate here offer convincing evidence of a three-way-link which ties cholinergic interneuron activity to changes in learning and behavior in a cocaine CPP extinction and links these to changes synaptic plasticity on MSNs.
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