Plasticity of afferent-specific synapses in the nucleus accumbens

By

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This thesis is dedicated to my mother, father, brother, and friends who have stood by me through this long journey. And to my late grandfather, Robert Hope, for always pushing me one step further.

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CHAPTER 1

An introduction to motivation: The nucleus accumbens as the brain's reward center

Sorting through the vast amount of environmental stimuli we see in a given day can at times be overbearing. Mundane sensations, such as that of the chair beneath us, are intermingled with more salient ones such as the seemingly ever present buzzing and dinging of our smartphones. Ultimately, our brains must sort through this barrage of sensation and come to an actionable conclusion. But how do we choose what to pay attention to, and what do we do with it? Multiple cortical and sub-cortical brain regions are responsible for parsing through incoming sensory stimuli while still others process information referencing our internal state of motivation and desire to obtain certain (sometimes) beneficial outcomes. These two neurological phenomena intersect at several integrative neuronal hubs giving rise to what is known as state dependent motivation. Regardless of the outcome of the action taken, consequent adaptations within these integrative hubs entail the cellular basis for learning and memory which ultimately shape our future responses to similar stimuli.

Based on the foundational research done by Pavlov and Skinner, studies in the past century examining the areas of the brain responsible for these learning processes, particularly reward-related motivated actions and reward association formation, have focused on outcome-based approaches coupled with targeted inhibition or stimulation of various neuronal regions. Several key brain structures now known to drive motivated choices and adaptive learning are localized within the mesolimbic dopamine system or the brain's reward pathway. In particular, one of the most prominent and well-studied regions within this pathway is nucleus accumbens (NAc). The

NAc is an integrative hub, combining neuromodulatory signals like dopamine and serotonin from the midbrain, glutamatergic inputs from various cortical and limbic regions, and local inhibitory transmission to transform environmental information to action initiation (Everitt and Robbins, 2005, 2016; Keeler *et al*, 2014). The NAc has been specifically focused on for its role in determining behavioral outcomes towards rewarding stimuli including palatable food or social gratification, as well as substances of abuse.

A seminal study performed in the 1950s first identified the role of the NAc and surrounding regions in reward-related behaviors (Olds and Milner, 1954). Olds and Milner implanted electrodes in the brains of rats near the NAc and median forebrain bundle and coupled activation of the electrodes to a lever in the rats' cage. They observed that the rats would readily press the lever to self-stimulate these regions while forgoing food and other stimuli. The authors concluded that this region of the brain must relay some aspect of hedonic value or pleasure and appropriately called it a 'reward center.' Subsequent research demonstrated that release of dopamine (DA) within the NAc coincides with reward acquisition (da Silva *et al*, 2018; Parker *et al*, 2016; Steinberg *et al*, 2013). Dopamine release in the NAc and other dopamine-innervated regions became a central idea in hypotheses detailing neurological mechanisms underlying reward association and seeking behaviors (Schultz, 2015). However, how the NAc itself utilized dopamine to gate the transformation of cortical and sub-cortical signals into various motivated behaviors was still unclear.

Since then, the NAc has become a central point of study with regard to reward-seeking behaviors, particularly maladaptive behaviors seen in drug addiction. As we now know, all abused classes of drugs result in an increase in dopamine within the NAc (Joffe *et al*, 2014). Psychostimulants such as cocaine can act directly in the NAc by blocking DA reuptake and

elevating local DA concentrations. Early identification of cocaine as a potent dopamine reuptake inhibitor drove researchers across the country to focus on how cocaine and other abused compounds affect the reward circuitry and perpetuate drug abuse (Ritz *et al*, 1987; Wise and Bozarth, 1987). Over time, the NAc was cemented as a critical brain region for the development of drug-seeking behavior using both pre-clinical rodent models (Everitt *et al*, 2016) and advanced imaging techniques in humans (Volkow and Morales, 2015). However, as the field of neuroscience advanced with ever increasing knowledge of the genetic, chemical, structural, and physiological diversity of cells throughout the brain, researchers have begun to appreciate the complexity of neurotransmission within the NAc and understand that dopamine release alone is insufficient to drive reward learning.

Overlapping with the discoveries of NAc dopaminergic signaling and its role in drug seeking, other researchers were identifying plasticity of excitatory (i.e. glutamatergic) synaptic connections induced by various neurotransmitter systems (Milner *et al*, 1998). Persistent bidirectional remodeling of synaptic connections by trafficking of glutamate receptors, modulation of their function via phosphorylation cascades, regulation of vesicular fusion by autoreceptors and feedback mechanisms, and the formation/deletion of new/extant synaptic connections became regarded as a cellular/molecular substrate of learning and memory (Lisman *et al*, 2012; Lisman and Zhabotinsky, 2001; Luscher and Malenka, 2012; Nabavi *et al*, 2014; Winder and Sweatt, 2001). These processes which determine glutamatergic synaptic strength are broadly referred to as synaptic plasticity and are ubiquitous throughout the brain. Subsequent questions arose as to how dopamine release within the NAc induced by cocaine or other abuse drugs might impact glutamatergic synaptic plasticity. It has since been shown repeatedly that these glutamatergic synapses are modulated by prior *in vivo* exposure to cocaine and other abused drugs (Chen *et al*,

2010; Kombian and Malenka, 1994; Malenka and Bear, 2004; Malinow and Malenka, 2002; Martin *et al*, 2006; Nicola *et al*, 2000). Over time, the modulation of NAc glutamatergic transmission rather than dopamine release became appreciated as the central substrate for the refinement of future behavioral paradigm selection (Belin *et al*, 2009; Everitt *et al*, 2005, 2016). As such, current investigation into the NAc circuit and reward driven behavior have centered on the modulation of specific glutamatergic synaptic inputs by dopamine and other neurotransmitters including serotonin, acetylcholine, opioids, oxytocin, and endocannabinoids.

We currently understand the NAc as a heterogeneous brain region comprised of multiple cell types that have several distinctive physiological features and functions. *In vivo* experience, be it with drugs of abuse, highly palatable food, or social interaction can induce some overlapping as well as several unique changes at these synaptic connections and reshape NAc function (Joffe *et al*, 2014). While it is not terribly surprising that unique stimuli evoke specific changes at the cellular and circuit level, these findings have necessitated ever-increasing degrees of scrutiny in order to understand the how complex neuronal adaptations drive specific behavioral changes. New technical approaches such as optogenetics and transgenic mouse lines are now enabling neuroscientists to further dissect the reward circuit and its ability to shape behavioral outcomes.

The research described herein strives to expand the model by which we understand the brain's ability to transform salient stimuli into new reward-associated behavioral paradigms by focusing on the ability of cocaine and non-drug rewards to modify the function of distinct glutamatergic inputs into the NAc. These endeavors are likely essential to the development of novel therapeutic approaches for treating motivational disorders, particularly drug abuse. Drug addiction and addiction-like behavioral disorders are debilitating mental conditions that affect millions worldwide and contribute to billions in medical costs each year in the United States alone.

Addiction is defined as a chronically relapsing disorder pertaining to the sustained intake of a harmful substance or harmful amounts of a mundane substance despite unwanted outcomes of such behaviors. Most treatment attempts fail due to the high rate of relapse following months or years of sobriety. As described in Chapter 2, this time-dependent increase in propensity for relapse is linked to persistent changes in the brain's reward circuitry and, as mentioned above, the modulation of glutamatergic synapses within the NAc.

The convergence of glutamatergic inputs from various brain regions including the basolateral amygdala (BLA), the medial prefrontal cortex (mPFC), the ventral subiculum of the hippocampus (vSub), and the medio-dorsal thalamus (MDT) in the NAc underlies the transformation of salient information into motivation towards the performance of reward-directed behaviors. In rodent models of drug-seeking and reinstatement, these inputs into the NAc have been shown to undergo significant changes regarding the efficacy of synaptic transmission and have each been linked to the formation and/or retention of drug-induced behaviors. At several of these inputs, drug-induced alterations of postsynaptic ionotropic glutamate receptor expression/function are well documented. However, there is an incomplete understanding of extrasynaptic and presynaptic regulatory mechanisms with respect to their impact on addiction-associated behaviors. The coupling of postsynaptic metabotropic glutamate receptors (mGlus) to endocannabinoid (eCB) signaling in presynaptic terminals within the NAc has been linked to psychostimulant induced behavioral adaptations. These receptors represent prominent targets for treating drug abuse and other motivational disorders.However, the NAc circuitry is complex partly due to the physiological heterogeneity implicit in these disparate inputs and the cell-types contained within the NAc itself. NAc medium spiny neurons (MSNs), which can be divided into two groups by their molecular and connective properties, drive seemingly opposing aspects of

reward behaviors (See Chapter 2). Each MSN subtype receives excitatory input from multiple glutamatergic afferents as well as local inhibitory and cholinergic neurons all of which contribute to determining MSN output and ultimately behavioral outcomes. As such, focused synaptic approaches are thus necessary to comprehend the role any given input plays in driving reward circuit function

Recent literature strongly suggests that plasticity of unique NAc glutamatergic connections engenders specific aspects of drug-induced behavioral adaptations as well as those associated with non-drug rewards. As of now, it is unknown how mGlu and eCB signaling modifies individual excitatory inputs and controls reward circuitry function. In order to understand how these signaling cascades influence synaptic transmission within the NAc and addiction-like behaviors, the underlying mechanisms must be investigated using an approach that is both input and cell-type specific. Using a combination of behavioral models of drug exposure, circuit- and cell type-specific synaptic physiology, and region-specific knockout approaches, the following work details a careful dissection of neuronal function in the NAc and connected brain regions to further the understanding of reward circuit function and drug abuse. I have hypothesized that mGlu and eCB function at distinct inputs into the NAc are discretely and temporally modulated by salient experience particularly exposure to cocaine. Herein, several transgenic mouse lines are used to deconstruct the physiological function of discrete NAc synaptic signaling cascades. Additionally, this work attempts to summarize interactions of NAc function with other forms of reward-seeking behaviors and their ripeness for future investigation. These findings add to an ever expanding model of experience-driven changes in the brain's reward circuitry, delineating several synaptic and molecular targets that can serve as the basis for future research into treatments for motivational disorders such as drug abuse.

CHAPTER 2

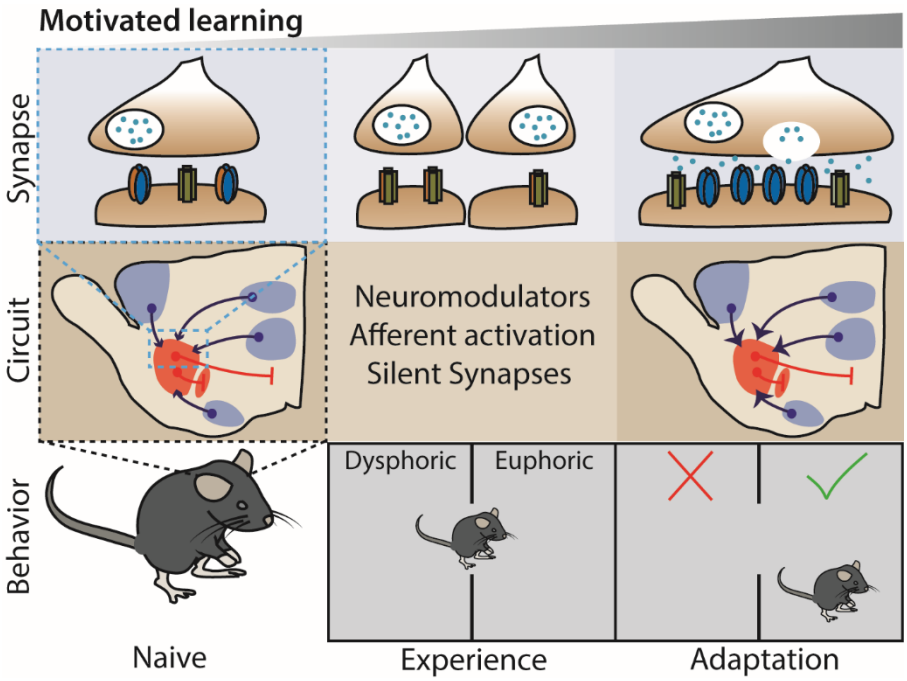
Synaptic transmission in Nucleus Accumbens: Lessons Learned from Experience

The following chapter was published as titled in *ACS Neuroscience*.

Turner BD, Kashima DT, Manz KM, Grueter CA, Grueter BA (2018). Synaptic Plasticity in the Nucleus Accumbens: Lessons Learned from Experience. ACS Chem Neurosci.

2.1 Abstract

Synaptic plasticity contributes to behavioral adaptations. As a key node in the reward pathway, the nucleus accumbens (NAc) is important for determining motivation-to-action outcomes. Across animal models of motivation including addiction, depression, anxiety, and hedonic feeding, selective recruitment of neuromodulatory signals and plasticity mechanisms have been a focus of physiologists and behaviorists alike. Experience-dependent plasticity mechanisms within the NAc vary depending on the distinct afferents and cell-types over time. A greater understanding of molecular mechanisms determining how these changes in synaptic strength track with behavioral adaptations will provide insight into the process of learning and memory along with identifying maladaptations underlying pathological behavior. Here, we summarize recent findings detailing how changes in NAc synaptic strength and mechanisms of plasticity manifest in various models of motivational disorders.



Graphical Abstract (legend not included in original manuscript) - Over the course of salient experience, regardless of stimuli presented, glutamatergic synapses within the NAc undergo significant modification following stimulus presentation in a given context. Following repeated pairings, environmental stimuli can drive behavior towards continued stimulus intake and is associated with other sensations induced by the stimulus presented *a priori.*

2.2 Introduction

The nucleus accumbens (NAc) is fundamental in driving goal-directed actions, integrating excitatory (glutamatergic) and neuromodulatory input along with local inhibitory control to optimize motivated behavioral outcomes. Long term changes in synaptic strength within the NAc underlies experience-dependent neural plasticity (Everitt *et al*, 2005; Voorn *et al*, 2004). These synaptic adaptations include intricate molecular epigenetic, biochemical, electrophysiological, and morphological changes in individual neurons, ultimately reshaping synaptic function (Volkow *et al*, 2011).

Fast excitatory synaptic transmission occurs through post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and n-methyl-D-aspartate (NMDA) ionotropic glutamate receptors. AMPA receptors are the primary contributor to excitatory synaptic transmission. Their trafficking in and out of the membrane is paramount to the process of post-synaptic plasticity. NMDA receptors, as well as metabotropic glutamate (mGlu) and other G-protein-coupled receptor (GPCRs), can initiate signaling cascades, affecting AMPA receptor surface expression and subunit composition throughout reward learning in an experience-dependent and temporally dynamic manner (Grueter *et al*, 2012; Huang *et al*, 2011b; Joffe *et al*, 2014; Kalivas, 2009; Kalivas *et al*, 2005; Luscher and Huber, 2010; Wolf, 2010). Many of these have been correlated or causally linked to motivational phenotypes in numerous models of developmental and psychiatric disorders. Maladaptive behaviors and the observed corresponding changes in NAc synaptic physiology are particularly well understood in models of addiction (Joffe *et al*, 2014; Koob, 2008, 2011), stress and depression (Bagot *et al*, 2015; Christoffel *et al*, 2015; Francis *et al*, 2015; Heshmati and Russo, 2015; Khibnik *et al*, 2016; Lim *et al*, 2012; Russo and Nestler, 2013), but are also hallmarks of eating disorders (DiLeone *et al*, 2012), schizophrenia

(Lee *et al*, 2015; McCollum and Roberts, 2015), pain perception (Schwartz *et al*, 2014) and autism spectrum disorders (Fuccillo, 2016; Rothwell, 2016). Because of the various contexts in which NAc synaptic plasticity is examined, creating a comprehensive model of the many plasticity mechanisms within this region has been elusive.

This chapter will attempt to summarize mechanisms known to reshape NAc excitatory synaptic transmission and how they are altered in model systems of psychiatric disorders. These include glutamate-mediated synaptic plasticity, signaling via serotonin, opioids, and endocannabinoids, as well as glial and astrocytic synaptic interactions. Additionally, this will attempt to highlight synaptic remodeling events that contribute to reward learning in healthy organisms and how these processes may serve as therapeutic targets for treatment of pathophysiologies underlying motivational disorders.

2.3 Anatomy of the NAc

As a key component of the mesolimbic dopamine (DA) system, the NAc is a functional interface between the limbic and motor systems responsible for bringing motivation to action (Mogenson *et al*, 1980). The NAc is a part of the ventral striatum comprised of shell and core subregions, which are thought to govern immediate responding to salient stimuli and conditioned reinforcement, respectively (Everitt *et al*, 2005). The NAc is predominantly (~90%) made up of GABAergic medium spiny neurons (MSNs) (Meredith, 1999; Sesack and Grace, 2010). MSNs, the output cells of the NAc, can be separated into one of two circuits distinguished by molecular, electrophysiological and anatomical properties (Grueter *et al*, 2013; Kupchik *et al*, 2015). Herein, we will identify the MSN subtypes based on their expression of the type-1 or type-2 dopamine receptors (D1 and D2 MSNs, respectively) (Lobo and Nestler, 2011), in which D1 MSNs largely project to the midbrain, while D2 MSNs project to the ventral pallidum (Smith *et al*, 2013).

However, it should be noted that this dichotomy is not as specific as the dorsal striatum as D1 MSNs can also project to pallidal brain regions (Kupchik *et al*, 2015). Recruitment of D1 or D2 MSNs has seemingly opposing effects on behavior: activation and activity of D1 MSNs corresponds with an increase in reward seeking and locomotion while activation of D2 MSNs promotes goal switching, catalepsy, and aversion (Bock *et al*, 2013; Lobo *et al*, 2010; Pascoli *et al*, 2015). However, recent findings using *in vivo* calcium imaging in the NAc and dorsal striatum indicate that these cells act in concert to drive motivated behaviors (Cui *et al*, 2013; Natsubori *et al*, 2017). Importantly, NAc MSNs are quiescent cells that rely on concerted excitatory drive from multiple glutamatergic afferents to elicit action potential generation, propagating information flow through the NAc circuit. Therefore, the strength and activity of these glutamatergic synapses determines the likelihood of afferent information being transformed to post-synaptic action potential propagation, making them vital nodes in defining overall circuit function.

Alterations in glutamatergic transmission engenders the integrative role the NAc plays in directing behavior. Glutamatergic brain regions that project to the NAc, such as medial prefrontal cortex (PFC; cognitive processing in goal-directed behavior), basolateral amygdala (BLA; conditioning forms of learning including processing of positive and negative emotions), ventral subiculum of the hippocampus (Hipp; contextual learning), and the dorsomedial thalamus (DMT; aversion, attention shifting), as well as co-release of glutamate from midbrain dopamine regions (Adrover *et al*, 2014; Morales and Root, 2014; Qi *et al*, 2016), are thought to encode salient information pertaining to proprioceptive self-assessments and externally available stimuli (Britt *et al*, 2012; Do-Monte *et al*, 2017; Everitt *et al*, 2005; Luscher and Malenka, 2011; Sesack *et al*, 2010; Stuber *et al*, 2012). By adjusting the strength of inputs from these afferent regions, the NAc is able to transform emotional and environmental information into action.

2.4 Neuromodulatory signals direct NAc circuit function

The strength of an afferent-MSN connection depends upon the number of release sites or synapses, the probability of vesicular release, and quantal size as determined by post synaptic receptor availability. In *ex vivo* electrophysiology studies, much of the observed changes occur via modifications to quantal size by modifying post-synaptic AMPA receptor populations or by alteration in release probability. Comparisons of current amplitude fluxed through AMPA and NMDA receptors, referred to as an AMPA/NMDA ratio, is a common metric for examining differences in synaptic strength across slices and conditions. This metric is often accompanied by direct measurement of quantal AMPA currents in the presence of tetrodotoxin (miniature EPSCs; mEPSCs) or replacing Ca^{2+} with strontium to evoke asynchronous EPSCs as a means to examine synaptic AMPA receptor populations. Additional analyses of isolated AMPA or NMDA receptor currents, including decay kinetics, current-voltage relationships, and coefficient of variation also provide insight into receptor subunit expression.

Synaptic plasticity of glutamatergic synapses can be initiated by numerous neurotransmitters, including glutamate itself. NMDA receptors are both ligand- and voltage-gated channels that act as coincidence detectors in the synapse (Paoletti *et al*, 2013). Entry of the second messenger Ca^{2+} through these receptors directs synaptic remodeling to strengthen or weaken future synaptic events (Luscher *et al*, 2012; Winder *et al*, 2001). In the NAc, NMDA signaling has been shown repeatedly to induce long term depression (LTD) of synaptic transmission reducing post-synaptic AMPA surface expression and/or function (**Fig 2.1C**). Long term potentiation (LTP) in the NAc is developmentally regulated (Schramm *et al*, 2002) and is sensitive to drug history (Pascoli *et al*, 2011), but has been difficult to evoke in *ex vivo* slice preparations. Regardless, NMDA activation is known to trigger LTP and LTD via signaling through ERK, PKC, or coupling

to CaMKII (Grueter *et al*, 2012; Joffe *et al*, 2014; Luscher *et al*, 2012). In *ex vivo* slice preparations, LTP and LTD can be evoked in the NAc by stimulation of glutamate release at high (100 Hz) (Kombian *et al*, 1994; Pascoli *et al*, 2011; Schramm *et al*, 2002; Yao *et al*, 2004) and low (1-13 Hz) frequencies, respectively (Grueter *et al*, 2010; Joffe and Grueter, 2016; Kombian *et al*, 1994; Ma *et al*, 2014; Pascoli *et al*, 2011; Robbe *et al*, 2002). LTP/LTD induction is often mirrored by bi-directional post-synaptic trafficking of AMPA receptors mediated in part by changes in scaffolding protein association and phosphorylation state (Malinow *et al*, 2002; Wolf, 2010; Yao *et al*, 2004). Transport of AMPA receptors and other proteins into the post synaptic density following LTP results in a restructuring of the synaptic spines (Bosch *et al*, 2014; Meyer *et al*, 2014; Nestler, 2013; Russo *et al*, 2010).

Both group-I (mGlu1/5) and group-II (mGlu2/3) metabotropic glutamate receptors are coupled to numerous signaling cascades that can exert pre- and post-synaptic effects (Cahill *et al*, 2014; Grueter *et al*, 2007; Niswender and Conn, 2010). Post-synaptic group-I mGlu receptors are G_q-coupled GPCRs that can initiate AMPA internalization (Grueter *et al*, 2010; Loweth *et al*, 2013; McCutcheon *et al*, 2011a) and/or an mGlu5 specific Ca^{2+}-dependent endocannabinoid (eCB) production in NAc MSNs (**Fig 2.1B**). eCBs can signal to pre-synaptic cannabinoid type-1 receptors (CB1Rs) (McCutcheon *et al*, 2011a; Robbe *et al*, 2002) or post-synaptic TRPV1 receptors (Grueter *et al*, 2010). Glutamate spillover following repeated vesicular fusion or glial-mediated release via cysteine-glutamate exchanger (xCT) and glial glutamate transporter (GLT-1) can also recruit pre-synaptic group-II receptors, which are $G_{i/o}$-coupled and decrease vesicular release probability (**Fig 2.1A**) (Knackstedt *et al*, 2009; Moussawi *et al*, 2009; Moussawi *et al*, 2011).

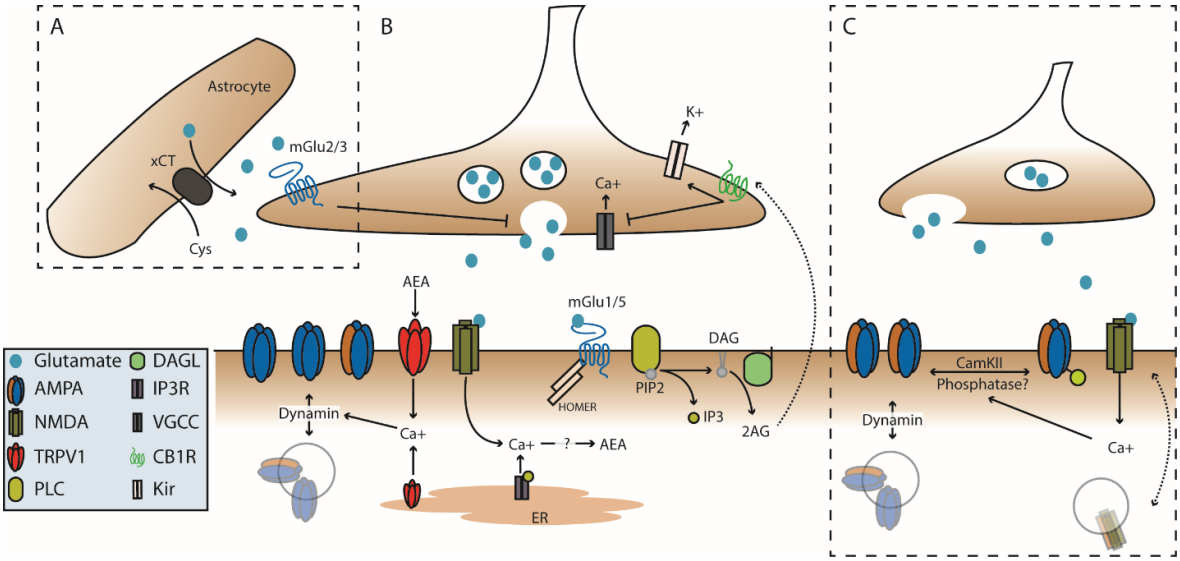


Figure 2.1 - mGlu and NMDA plasticity mechanisms coordinate pre- and post-synaptic function of NAc glutamatergic synapses. **A.** Extra-synaptic glutamate homeostasis couples to group-II mGlu activation. Extra-synaptic glutamate is tightly regulated by astrocytic cysteine-glutamate antiporter (xCT). High extracellular glutamate activates group-II mGlu receptors to decrease presynaptic release. **B.** Post synaptic activation of group-1 mGlu receptors is known to recruit PLC to generate IP3 and DAG. DAG can be further cleaved by DAGL to a free fatty acid and 2-arachidonyl glycerol (2AG), which can signal to presynaptic CB1Rs. Activation of CB1Rs can act via inhibition of VGCCs and/or activation of pre-synaptic potassium channels to decrease vesicular release. Additionally, activation group-1 mGlu receptors can also induce a calcium dependent synthesis of anandamide (AEA), which likewise acts on CB1Rs but also activates TRPV1 channels. Activation of TRPV1 at the membrane or on the ER induces a dynamin-dependent internalization of AMPA receptors. **C.** NMDA dependent LTD and LTP. Endogenous glutamate/glycine binding and concurrent depolarization activates NMDA receptors allowing an influx of calcium which can couple to downstream phosphatase/kinase cascades. These likely include CaMKII and calcineurin, which can phosphorylate/dephosphorylate AMPA receptors, respectively. This contributes to their insertion or removal from the postsynaptic density. However, this mechanism is not well defined in the NAc.

Serotonin

The NAc receives extensive inputs from the dorsal raphe nucleus (DRN), a mesencephalic structure rich in serotonin (5-HT)-containing perikarya (Brown and Molliver, 2000). Consistent with the appositional relationship between 5-HT fibers and afferent synaptic inputs (Soghomonian *et al*, 1989), 5-HT has been shown to induce LTD of excitatory synaptic strength onto MSNs (Burattini *et al*, 2014; Dolen *et al*, 2013; Mathur *et al*, 2011; Muramatsu *et al*, 1998). This form of LTD (5-HT-LTD) is expressed at a pre-synaptic locus and mediated predominately via the 5-HT1B receptor, a $G_{i/o}$-coupled GPCR implicated in reward-related behavior (**Fig 2.2**). Low frequency stimulation (LFS) has also been shown to trigger 5-HT-LTD in a CB1R-dependent manner (Best and Regehr, 2008; Burattini *et al*, 2014), indicating eCBs are downstream of 5-HT signaling and may function cooperatively to regulate NAc circuit dynamics.

The 5-HT1B receptor also mediates oxytocin (OT)-induced synaptic adaptations in the NAc. OT is a neuropeptide implicated in neuropsychiatric conditions featuring maladaptive social behavior, including autism and schizophrenia (Dolen *et al*, 2013). *Ex vivo* bath-application of OT induces robust LTD of EPSCs onto D1 and D2 MSNs in the NAc that is blocked by NAS-181, a selective 5-HT1B receptor antagonist (Dolen *et al*, 2013). These data indicate that OT-mediated 5-HT release in the NAc triggers a form of pre-synaptic LTD that is required for social reward behavior.

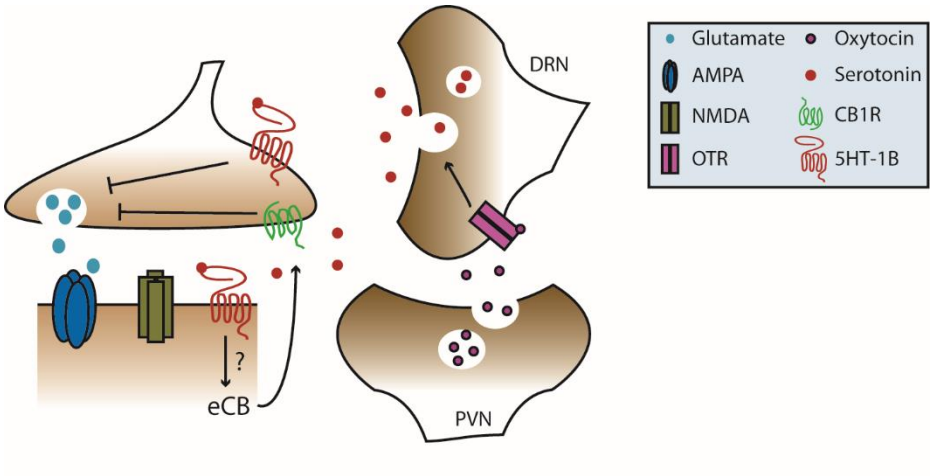


Figure 2.2 - Oxytocin gates serotonergic LTD in the NAc. Oxytocin release from paraventricular nucleus (PVN) terminals drives release of 5-HT from dorsal raphe (DRN) afferents. 5-HT in the NAc may act on either pre-or post-synaptic 5HT-1B receptors, which can either directly inhibit neurotransmitter release or indirectly through eCB signaling. Beyond several isolated studies, how 5-HT modifies NAc excitatory transmission is unknown.

Opioids

Opioids are a widely expressed peptidergic modulatory system affecting neuronal function and circuity dynamics. The opioid system consists of four receptor subtypes (mu, delta, kappa, and opioid receptor like-1) and three endogenous ligands (endorphin, enkephalin, and dynorphin) with varying degrees of ligand specificity and expression patterns (Al-Hasani and Bruchas, 2011). Within the NAc, D1 and D2 MSNs express endogenous opioids in a similarly dichotomous manner: D1 MSNs primarily express dynorphin (Dyn) and D2 MSNs expressing encephalin (Enk), with striatal systems being largely described to lack beta-endorphin (Khachaturian *et al*, 1985). However, opioid receptors (ORs) are broadly expressed and how they regulate excitatory transmission in the NAc remains obscure (Bruchas *et al*, 2010; Chartoff and Connery, 2014; Lutz and Kieffer, 2013).

Activation of mu, delta, or kappa ORs can drive "liking" or "wanting" behavioral outcomes in a manner dependent on NAc subregion (Castro and Berridge, 2014). While *in vivo* studies have been abundant, less is known how these receptors control synaptic transmission. In the dorsal striatum, where MSNs are more discretely subdivided anatomically into a 'patch' and 'matrix' framework, mu ORs are found uniquely expressed in patches and are activated by enkephalin to decrease microcircuit inhibition and promote MSN activation (Banghart *et al*, 2015). In the NAc, activation of mu ORs decreases NMDA and AMPA receptor currents with little effect on membrane properties (Martin *et al*, 1997) with functional expression both pre- and post-synaptically (**Fig. 2.3**) (Chartoff *et al*, 2014). It has been demonstrated mu ORs exert strong control over thalamic but not motor cortex inputs into the dorsal striatum (Atwood *et al*, 2014), but it is unclear whether NAc MSNs are under similar mu OR control. These findings suggest mu-OR signaling in the NAc may similarly be separable by afferent origin and should be investigated

accordingly.

A more recent report demonstrated that stimulation of Dyn+ NAc MSNs drives both reward and aversion via stimulation of the dorsal and ventral NAcSh, respectively, and is dependent on kappa OR signaling (Al-Hasani *et al*, 2015). Kappa ORs specifically decrease excitatory drive of BLA but not VH inputs onto D1 MSNs and decrease inhibitory drive onto D2 MSNs. Thus, kappa OR activation within the NAc results in increased transmission from the VH and BLA through D1 MSN activation (Tejeda *et al*, 2017). It should be noted that kappa-OR signaling can also inhibit DA signaling by inhibiting VTA terminals (Muschamp and Carlezon, 2013). Beyond these few reports, mechanistic details of OR function in the NAc are largely unexplored.

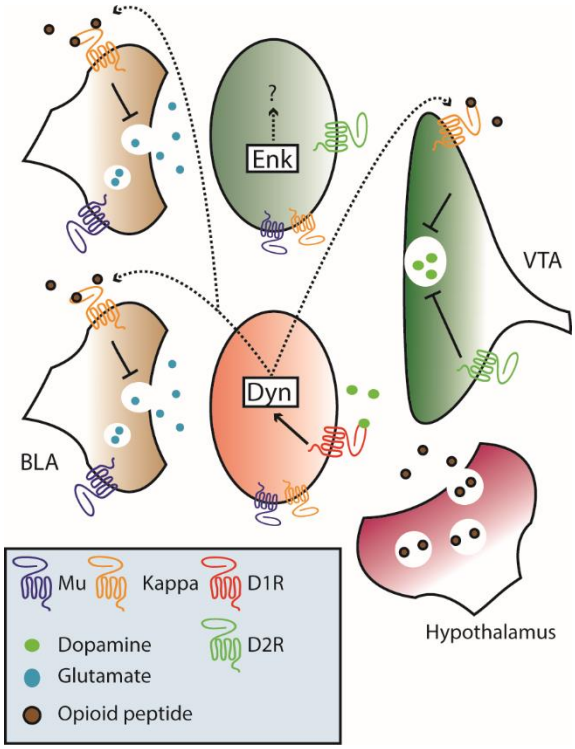


Figure 2.3 - Endogenous opioids regulate synaptic transmission in the NAc. Opioid receptors are expressed widely on glutamatergic terminals and cell bodies within the NAc. Mu-ORs likely function pre-synaptically and reduce release probability. Kappa-ORs are expressed on glutamatergic afferents (excluding the vHipp) and inhibit neurotransmitter release, particularly onto D1 (red) MSNs. Kappa-ORs are activated by Dyn which is produced locally by D1 MSNs. Dyn can also inhibit dopamine release by acting on VTA terminals kappa-ORs. Activation of D1 receptors promotes pro-dynorphin expression, serving to inhibit glutamatergic drive onto these MSNs. While Dyn and Enk are produced by NAc MSNs, opioid peptides can also be released from hypothalamic projections from the Arcuate nucleus and the Lateral Hypothalamus.

Endocannabinoids

The cannabinoid 1 receptor (CB1R) is implicated in substance abuse disorders (Clarke *et al*, 2013b; Hirvonen *et al*, 2012; Schacht *et al*, 2012; Zuo *et al*, 2009). CB1Rs are the most abundant G-protein coupled receptor in the CNS and are localized mainly at pre-synaptic glutamate and GABA terminals in select neuronal populations (Kano *et al*, 2009). CB1Rs are activated by Delta-9-tetrahydrocannabinol, (Δ^9-THC), the main psychoactive substance in *Cannabis sativa*. Endogenous cannabinoids are produced via post-synaptic *de novo* synthesis with subsequent release and retrograde activation of pre-synaptic CB1Rs. In the NAc, stimulation of mGlu5 receptors leads to a rise in postsynaptic calcium. This in turn leads to retrograde signaling through eCB release and activation of presynaptic CB1 receptors. Activation of CB1Rs reduces neurotransmitter release by decreasing release probability in a presynaptic K+ channel dependent manner (**Fig 2.1**) (Grueter *et al*, 2010; Hoffman and Lupica, 2001; Ohno-Shosaku and Kano, 2014; Robbe *et al*, 2003; Robbe *et al*, 2002). Contrary to the dorsal striatum, CB1Rs are not expressed in NAc MSNs but are expressed by NAc fast-spiking interneurons and on glutamatergic terminals (Winters *et al*, 2012).

2-arachidonylglycerol (2-AG) is the primary eCB mediating retrograde eCB signaling and is synthesized from diacylglycerol precursors by diacylglycerol lipaseα (DAGLα) in the adult brain. The eCB anandamide (AEA), in addition to CB1R activation, can also activate TRPV1 channels (Kauer and Gibson, 2009; Ramsey *et al*, 2006; Renteria *et al*, 2014; Zygmunt *et al*, 1999). TRPV1 is a nonselective cation channel that is highly permeable to calcium and is activated by acidic pH, high temperature and specific lipid species (Kauer *et al*, 2009; Ramsey *et al*, 2006). TRPV1 function is commonly associated with pre-synaptic mechanisms including a form of LTD triggered by post-synaptic group I mGlu receptors at excitatory synapses on interneurons in the

hippocampus (Gibson *et al*, 2008). However, TRPV1 activation can also act post-synaptically to induce depression of excitatory synapses in the NAc core (Grueter *et al*, 2010). This adds to the eCB system's canonical role in regulating pre-synaptic release and positions it as a versatile modulator of NAc circuit function.

Glial regulation of drug-reward learning

In addition to neuron-centric mechanisms of synaptic and behavioral plasticity, a growing body of research points to the importance of glia and the immune system. Specifically, microglia and astrocytes are increasingly found to play active roles in sculpting synaptic physiology and behavior. Microglia are the brain's resident macrophage (Prinz and Priller, 2014). These cells make up 10% of the brain parenchyma (Kettenmann *et al*, 2013) and play a key role in mediating immune responses in this region (Joseph and Venero, 2013). Microglia play an important role in development, learning, and brain homeostasis (Bilbo and Schwarz, 2012) by refining learning-induced spine formation as well as synaptic pruning (phagocytosis) (Parkhurst *et al*, 2013; Schafer *et al*, 2012). In the context of drug-reward learning, the function of these cells appears complex and sometimes contradictory.

Importantly, microglia also influence synaptic function in the NAc. Microglia in the NAc express toll like receptor 4 (TLR4) (Kashima and Grueter, 2017; Schwarz *et al*, 2013), a pattern-recognition receptor of the innate immune system that detects bacterial lipopolysaccharide (Bohannon *et al*, 2013) and endogenous "danger signals" such as those produced during an inflammatory response (O'Neill, 2008). TLR4 knockout mice lack NMDA-dependent LTD in NAc core linking the immune system with synaptic plasticity (Kashima *et al*, 2017). Beyond TLR4, microglia play a role NAc synaptic physiology and may mediate aspects of drug reward susceptibility. Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine upregulated in

many conditions including after activation of TLR4 (Bohannon *et al*, 2013). Microglial TNFα decreases synaptic strength as measured by AMPA/NMDA ratios the NAc D1 MSNs to oppose synaptic and behavioral changes brought about with non-contingent cocaine exposure.(Lewitus *et al*, 2016). These findings provide compelling evidence for the immune system facilitating and perhaps driving adaptations in NAc excitatory transmission.

Besides microglia, astrocytes play major roles in sculpting physiology and behavior (Clarke and Barres, 2013a). In the NAc, astrocytes are capable of regulating the concentration of extrasynaptic glutamate via the cysteine-glutamate exchanger (catalytic subunit = xCT) and GLT-1, which regulate extracellular glutamate levels. GLT- 1 is expressed on astrocytes and is responsible for glutamate uptake. Alterations in GLT-1 function can thus have profound impact on synaptic glutamate signaling (Knackstedt *et al*, 2009; Knackstedt *et al*, 2010). N-acetylcysteine, which stimulates xCT, bi-directionally regulates EPSC amplitude in NAc MSNs; low doses (0.5μM) decreases pre-synaptic release probability in a group-II mGlu dependent manner while high doses (50μM) increase EPSC amplitude in via mGlu5 activation (Kupchik *et al*, 2012). The increase in extracellular glutamate acts on neuronal pre-synaptic mGlu2/3 to decrease vesicular release probability (Kalivas, 2009).

2.5 Experience reshapes NAc synapses and plasticity mechanisms

Experimentally, acute slice physiology has been instrumental in elucidating mechanisms of synaptic plasticity in the NAc. Importantly, *in vivo* experience can also drive new synapse formation, strengthen or weaken select afferent inputs, and impede or enhance molecular plasticity mechanisms. Such stimuli include those used in models of motivated appetitive behaviors, anxiety, and depression (Richard *et al*, 2013). From the seminal work of Thomas et al 2001, which defined a correlational change in NAc MSN synaptic strength following cocaine exposure, investigating

adaptations in synaptic function in acute slices following *in vivo* experience has led to developments in recent years showing a causal effect of synaptic plasticity and altered behavioral outcomes (Conrad *et al*, 2008; Lee *et al*, 2013; Loweth *et al*, 2014a; Ma *et al*, 2014; Pascoli *et al*, 2014; Pascoli *et al*, 2011; Zhu *et al*, 2016). As addressed below, this powerful approach has repeatedly demonstrated a functional relationship between glutamatergic synaptic strength and behavioral plasticity. As such, NAc synaptic plasticity has become nearly inseparable from questions interrogating reward and motivation. By focusing on the plasticity mechanisms within the NAc rather than the various psychiatric disease models, we aim to elucidate common mechanisms by which *in vivo* experiences drive change in the NAc circuit.

AMPA receptor expression and function coincides with in vivo experience

Expression and function of AMPA and NMDA receptors in the NAc are strongly associated with experience-dependent behavioral plasticity, particularly in drug abuse models (Luscher *et al*, 2011; Russo *et al*, 2010). Thomas *et al* demonstrated NAc shell MSNs have a reduced AMPA/NMDA ratio following repeated drug exposure and is concurrent with a reduction in NMDA-dependent LTD (Thomas *et al*, 2001). This phenomenon was then shown to be mediated by the challenge dose of cocaine/saline administered prior to the recording (Boudreau *et al*, 2007). Thus, AMPA/NMDA ratios are decreased immediately following drug, but are strengthened following a short abstinence period and can be reduced again with re-exposure (Kourrich *et al*, 2007). These findings demonstrated a temporal restructuring of glutamatergic signaling within the NAc following salient experience. Similar results with calcium-permeable AMPA receptors (CP-AMPA) have been demonstrated following drug self-administration, leading AMPA-receptor expression and function to be thought of as a neural correlate of incubation of drug craving (Conrad *et al*, 2008). However, it should be noted that the contingency of drug delivery determines the type

of remodeling seen in the NAc with respect to AMPA subunit composition (McCutcheon *et al*, 2011b) but both favor an increase in glutamatergic drive.

Synapse maturation is a developmental process underlying neural circuit formation and is considered a critical physiological substrate for learning and memory (Brown *et al*, 2011; Huang *et al*, 2009; Kerchner and Nicoll, 2008). In the NAc, the relative abundance of silent or AMPA receptor deficient synapses is increased following acute cocaine self-administration (**Fig 2.4A**). These nascent synapses are short-lived and mature over time via insertion GluA2-lacking CP-AMPA receptors (**Fig 2.4B**) (Brown *et al*, 2011; Conrad *et al*, 2008; Huang *et al*, 2009; Lee and Dong, 2011; McCutcheon *et al*, 2011b). Maturation occurs following several weeks after drug withdrawal and requires PSD95 and SAP102 MAGUK proteins (Shukla *et al*, 2017). The generation of CP-AMPA containing synapses is correlated with incubation of drug seeking in self-administration models (Loweth *et al*, 2014b) and is not normally seen following non-contingent drug administration. However, recent work has demonstrated increases in AMPA rectification, a measurement CP-AMPA expression, following repeated non-contingent exposure. Short access to cocaine self-administration drove CP-AMPA expression at PFC-D1 synapses, while long-access, presumably resulting in enhanced negative withdrawal symptoms, drove CP-AMPA expression at D2 MSNs specifically at BLA synapses (Terrier *et al*, 2016). Additionally, some synapses, such as those from the DMT-NAc, are reported to contain a high density of CP-AMPA at baseline which is unaffected by drug history. However, the formation and maturation of silent synapses is seen at this input, suggesting maturation may proceed by a non CP-AMPA mechanism (Neumann *et al*, 2016). Notably, increases in mature spine number are also seen in non-contingent exposure paradigms and are specific for D1 MSNs (Kim *et al*, 2011; MacAskill *et al*, 2014).

While both NMDA and mGlu plasticity described above are initiated through local signals

within the dendritic spine, transcription/translational changes are required to maintain the effect (Russo *et al*, 2013; Scheyer *et al*, 2014). Such changes include altered expression of Homer1a, CREB, and ΔfosB (Brown *et al*, 2011; Grueter *et al*, 2013; Szumlinski *et al*, 2008). Salient experience is also coupled to upregulation of transcription factors in the NAc that can alter AMPA/NMDA expression. Two well studied transcription factors, CREB and ΔfosB, are recruited following cocaine exposure and are sufficient to drive changes in synaptic transmission. CREB is expressed following salient experience and can drive behavioral responding to both aversive and rewarding stimuli (Barrot *et al*, 2002). Cocaine-induced or viral-mediated overexpression of CREB alters membrane and synaptic properties of NAc MSNs (Brown *et al*, 2011; Dong *et al*, 2006). Likewise, ΔfosB is upregulated in the NAc following exposure to abused drugs (Nestler, 2008; Robison and Nestler, 2011) and is associated with behavioral adaptations tied to addiction. Interestingly, overexpression ΔfosB in the NAc 'silences' D1 synapses in the shell and core but may unsilence D2 MSN synapses via AMPA insertion in the NAc shell. Notably, ΔfosB promotes the expression of GluA2 as well as CaMKII, and these effects are restricted to D1 MSNs in the NAc (Kelz *et al*, 1999; Robison *et al*, 2013; Vialou *et al*, 2010). As such, ΔfosB is positioned as a critical transcriptional regulator of cocaine-induced synaptic adaptations in the NAc.

Alterations in NAc glutamatergic transmission are not limited to drug-contexts. Interestingly, appearance of mature CP-AMPA containing synapses is also observed days after removing animals from a highly palatable 'junk-food' diet (**Fig 2.4C**) (Oginsky *et al*, 2016), suggesting palatable food and 'natural' rewards may be a more potent driver of this adaptation. This may serve to increase appetitive drive for palatable food, as inhibiting glutamatergic transmission via intra-accumbens infusion of CNQX, an AMPA receptor antagonist, stimulates voracious feeding behavior (Maldonado-Irizarry *et al*, 1995).

Additionally, models of depression and anxiety induced by stressors also drive remodeling of NAc glutamate synapses (Russo *et al*, 2013). Chronic restraint stress has been shown to impair the induction of LTD within the NAc core via an MC4R-dependent signaling cascade (Lim *et al*, 2012). This is mediated by a selective internalization of GluA2-*containing* receptors resulting in an unmasking of synaptic GluA2-lacking, Ca^{2+} permeable AMPA receptors selectively at D1 MSNs. Chronic social defeat stress results in a decrease in mEPSC frequency at D1 MSNs but an increase in synaptic events at D2 MSNs. Chronic pain, which likewise induces an amotivational phenotype, caused a decrease in AMPA/NMDA ratios at D2 MSNs. This is in part mediated by increased GluN2B-subunit expression but an abolition of NMDA mediated LTD at D2 synapses (Schwartz *et al*, 2014). Similarly, precipitated withdrawal from morphine, which induces conditioned place aversion, selectively strengthened DMT-NAc D2 synapses and coincided with an increase in AMPA rectification (Zhu *et al*, 2016). Thus, the canonical model of increased synaptic connectivity at D1 and D2 MSNs promoting reward and aversive behavior may be incomplete, as these adaptations coincide with both circumstances.

NMDA function and receptor-dependent plasticity induction

NMDA receptors are implicated in experience-dependent synaptic changes. Several studies have demonstrated that NMDA receptor activation correlates with drug-induced synaptic changes (Beutler *et al*, 2011; Cahill *et al*, 2014). GluN2B-containing NMDA receptors are of particular importance to experience-driven plasticity in the NAc. GluN2B receptors have much slower deactivation kinetics, resulting greater net ion flux and Ca^{2+} entry upon glutamate binding and depolarization. These large currents extend the temporal binding window that allows coupling of synaptic events to neuronal firing (Paoletti *et al*, 2013). GluN2B receptors are found in high concentrations throughout the developing brain and facilitate formation of new synaptic

connections via their high concentration in silent synapses (Kerchner *et al*, 2008). The *de novo* generation of NAc silent synapses in adults occurs following acute withdrawal from drug self-administration and coincides with an increase in the relative expression of GluN2B (Huang *et al*, 2009). The formation of new synapses and their subsequent maturation (see above) suggests an increase in connectivity between glutamatergic afferent regions and the NAc following salient experience, increasing their influence on MSN activation. Importantly, the formation of these synapses has been demonstrated at specific afferent-NAc connections including the BLA (Lee *et al*, 2013), PFC (Ma *et al*, 2014), and DMT (Neumann *et al*, 2016), demonstrating their prevalence in NAc circuit remodeling. Thus, GluN2B NMDA receptors are crucial for forming new synapses in response to in vivo experience.

NMDA receptors are also crucial for directing synaptic strength. Following non-contingent drug exposure, NMDA currents from the DMT are selectively enhanced via increase in GluN2C/D (Joffe *et al*, 2016). The increase of NMDA function in cocaine treated animals also unmasked an NMDA-dependent LTD at D1 MSN synapses. In line with this finding, resetting glutamatergic inputs from specific brain regions by inducing NMDA-dependent plasticity *in vivo* at specific inputs can diminish relapse like behavior in rodents. NMDA-dependent LTD of BLA-NAc synapses reduced cue-primed reinstatement to drug seeking (Lee *et al*, 2013). An LTD protocol at vHipp-NAc synapses, previously shown to be NMDA dependent, disrupted preference for the drug-paired lever in a cue-induced reinstatement task (Pascoli *et al*, 2014). Additionally, a NMDA-dependent LTD protocol of PFC-NAc synapses reduced locomotion in a cocaine-induced locomotor sensitization (Pascoli *et al*, 2011). However, the same *in vivo* NMDA-dependent LTD protocol was impaired selectively at PFC-D1 synapses in *ex vivo* slice preparations from mice withdrawn from cocaine self-administration and failed to reduce responding for the active lever in

a cue induced reinstatement task(Pascoli *et al*, 2014). Yet others have shown the same LTD protocol of PFC-NAc synapses also required mGlu1, was only present in animals withdrawn from cocaine, and was able to reduce cue-induced reinstatement in rats (Ma *et al*, 2014). These findings suggest that NMDA-dependent LTD is able to ameliorate motivated behavior in experienced animals. The nuanced differences in the models and results indicate additional studies are necessary to clarify the impact of salient drug experience on NMDA signaling at specific synapses.

Recent findings demonstrate that global TLR4 knockout mice lack NMDA-LTD in the NAc core and display reduced drug-induced locomotion and place preference (Kashima *et al*, 2017). Importantly, there is evidence suggesting that pharmacologic antagonism of TLR4 attenuates drug reward learning to both opioids (Hutchinson *et al*, 2012) and cocaine (Northcutt *et al*, 2015). Such findings led to the idea that drugs of abuse directly interact with TLR4 to induce cellular changes (Bachtell *et al*, 2015). Also implied is that TLR4 is necessary for drug-reward learning. However, there is controversy surrounding some of these points (Tanda *et al*, 2016). These findings suggest a link between TLR4 expressing microglia and drug-induced adaptations in NAc NMDA plasticity.

mGlu plasticity shifts post-synaptically following drug experience

mGlu function is negatively impacted by both acute and chronic exposure to cocaine (**Fig 2.4B, C**). Many studies have demonstrated the induction of mGlu5-dependent LTD is blunted following a single or repeated cocaine experience [for examples see (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; Grueter *et al*, 2007; Loweth *et al*, 2013; McCutcheon *et al*, 2011a; Robbe *et al*, 2002; Shin *et al*, 2015)]. Cocaine-induced abolition of mGlu LTD is thought to be mediated by changes in structural protein Homer isoforms. Homer1a expression induced by acute cocaine exposure sequesters mGlu5 from the membrane surface but increases mGlu1 (Szumlinski *et al*,

2006; Szumlinski *et al*, 2008). This switch in synaptic control from mGlu5 to mGlu1 is proposed to change downstream plasticity targets to favor CP-AMPA internalization over eCB production (McCutcheon *et al*, 2011a; Szumlinski *et al*, 2006; Szumlinski *et al*, 2008). This is consistent with the finding that mGlu dependent eCB production is altered in rodents exposed to cocaine, but this is not due to changes in CB1R expression/function (Fourgeaud *et al*, 2004; Robbe *et al*, 2002).

Much like NMDA-LTD, putative mGlu dependent plasticity is also modified in a synapse specific manner. Withdrawal from drug self-administration unmasked an mGlu1 and NMDA dependent LTD at PFC-NAc shell MSNs (Ma *et al*, 2014). Likewise, a separate LTD protocol previously shown to be mGlu-dependent was capable of evoking LTD at PFC-NAc shell synapses and was enhanced in mice that administered cocaine (Pascoli *et al*, 2014). *In vivo* induction of this putative mGlu-LTD at PFC synapses also ameliorated cue-induced drug seeking. Notably, mGlu1 PAMs infused into the NAc are able to achieve a similar effect in a cue-induced reinstatement task (Loweth *et al*, 2014a). Additionally, recent reports have demonstrated an mGlu1 positive allosteric modulator (PAM) can "reset" CP-AMPA containing synapses in the NAc while also reducing drug induced place preference (Shukla *et al*, 2017). Given the expression of mGlu1 LTD at PFC synapses in cocaine exposed mice and the efficacy of *in vivo* PFC mGlu-LTD, it is possible that the ability of intra-NAc PAM infusion to also reduce drug seeking is mediated via action on PFC terminals. Because of this, it may be of interest for future studies to focus on experience-induced alterations in group-I mGlu-dependent plasticity in a synapse-specific manner.

In addition to group-I mGlu function, signaling through group-II mGlu receptors are also heavily tied to drug experience and are coupled to glutamate homeostatic regulation by astrocytes (Kalivas and Volkow, 2011; Moussawi *et al*, 2011). Notably, extracellular glutamate is elevated following drug exposure in self-administering animals (McFarland *et al*, 2003). These changes in

extracellular glutamate concentrations arise via downregulation of NAc astrocytic xCT (**Fig 2.4C**). Withdrawal from cocaine and nicotine downregulates xCT (Knackstedt *et al,* 2009; Scofield and Kalivas, 2014). In similar studies, multiple drugs of abuse have been shown to downregulate GLT-1, which can be pharmacologically rescued by Ceftriaxone (Knackstedt *et al*, 2010). This results in increased neuronal pre-synaptic release probability promoting increased glutamatergic transmission for drug-related signals/cues leading to relapse (Kalivas, 2009). Increasing astrocyte activity using Gq-coupled designer-receptor exclusively activated by designer drugs (DREADD) resulted in increased extracellular glutamate and was associated with decreased cue-induced reinstatement in rats (Scofield *et al*, 2015).

mGlu and NMDA signaling seem to be differentially recruited throughout reward learning in animal drug-exposure models. However, it is yet unclear how these changes result in altered circuit function. For one, the multitude of experimental paradigms, including rodent model, behavioral setup, and cell-type/input specificity, obfuscate comparisons across studies. Additionally, it is apparent that contingent and spatial recognition are more efficient at driving change in the NAc circuitry than context association or home cage experiences in rodents. However, there is an emerging trend suggesting salient experience induces an increased susceptibility to NMDA- and mGlu1-dependent AMPA internalization and a reduction pre-synaptic control by group-II mGlu and mGlu5-dependent eCB signaling.

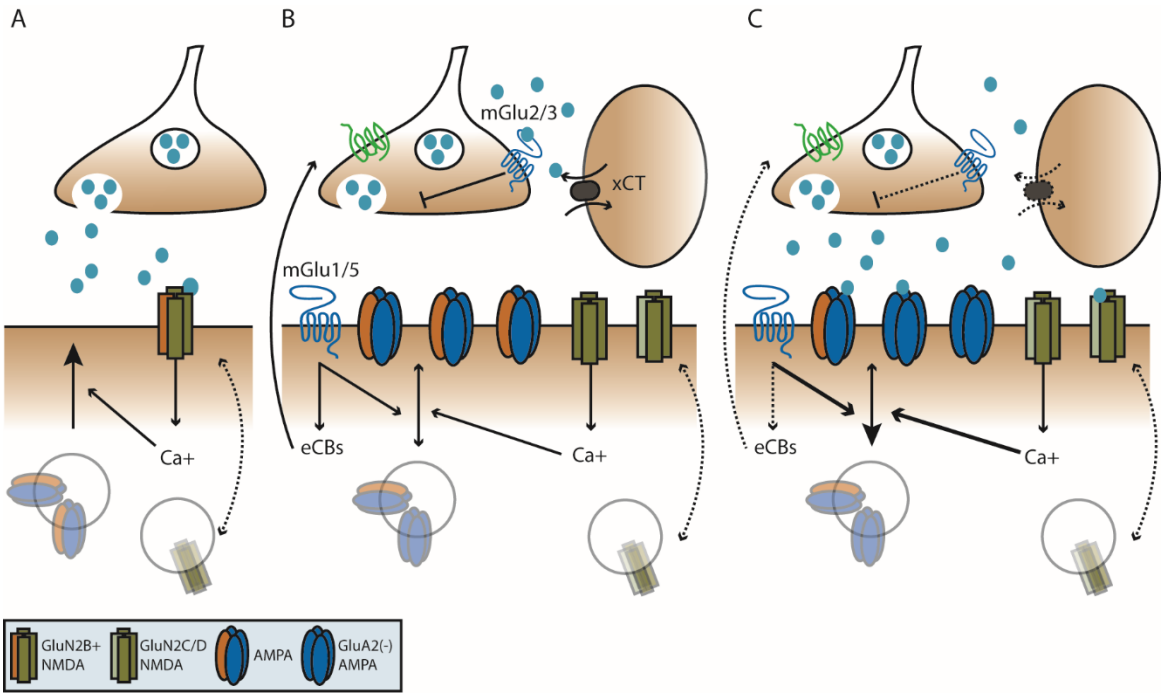


Figure 2.4 - Experience drives plasticity of NAc glutamatergic transmission *in vivo*. **A.** Following salient experience, nascent 'silent' synapses are formed in the NAc, lacking functional AMPA receptors but expressing high concentrations of GluN2B-NMDA receptors. **B.** As these synapses mature, they are under the control of mGlu and NMDA-dependent plasticity mechanisms. These mechanisms are extensively observed in mature, experience-naïve animals. **C.** Following extensive abstinence from the initial salient experience, such as chronic stress, drug self-administration, or after acute removal from highly-palatable chow, GluA2-lacking AMPA receptors are more abundant in the post-synaptic density. In cocaine-specific contexts, GluN2C/D NMDA receptors are found in a higher concentration in a subset of synapses. Broadly, group-1 mGlu-dependent eCB signaling is decreased and lowered concentrations of extra-synaptic glutamate stemming from astrocytes leads to decreased group-II mGlu receptor inhibition of vesicular release. Additionally, NMDA and group-1 mGlu activation favor internalization of AMPA receptors.

Neuromodulatory regulation of NAc synapses following salient experience

The majority of studies investigating changes in glutamatergic NAc signaling have focused on AMPA, NMDA, and mGlu receptors. However, the various modulatory signals that interact with these receptors can also be impacted by salient experience. While the role of 5-HT in drug-related behavior remains enigmatic, 5-HT1B activity has been shown to contribute to the reinforcing properties of psychostimulants, including cocaine and amphetamine (Barnes and Sharp, 1999; Fletcher *et al*, 2002). 5-HT-LTD in the NAc is impaired for up to 72 hours following a single *in vivo* administration of cocaine, an effect rescued by a membrane-permeable PKA inhibitor (Burattini *et al*, 2014).

Similarly, opioids and ORs within the NAc are impacted by salient experience and can drive behavior. Repeated force swim stress induces a kappa-opioid dependent ERK1/2 phosphorylation within the NAc (Bruchas *et al*, 2008). Additionally, stress induces phosphorylation of kappa ORs (Land *et al*, 2008) consistent with their and dynorphin's role in stress-induced behaviors (Muschamp *et al*, 2013). Dyn signaling is also implicated in models of drug abuse (Shippenberg *et al*, 2007) but only recently has a synaptic phenotype been demonstrated. Cocaine exposure can selectively impair dynorphin-A induced LTD of glutamatergic synapses with no effect on inhibitory transmission (Mu *et al*, 2011). Additionally, these authors found that dynorphin-B exhibited non kappa-OR dependent effects that were unaffected by cocaine. As previous work has focused extensively on Dyn and kappa OR signaling, future studies should focus on delta and mu ORs synaptic function and how they are impacted by salient experience.

Endocannabinoid signaling is also tied to salient experience, although this is in part due to its known dependence on mGlu signaling in the NAc. While CB1R activation is important for the

expression of these behavioral phenotypes, few studies have identified changes in the receptor or eCB synthetic enzymes. Of note, stimulant exposure impacts mGlu-dependent eCB production but leaves CB1R function intact (Fourgeaud *et al*, 2004). However, acute exposure to the CB1R agonist THC results in a desensitization of the receptor and blunts eCB-LTD (Mato *et al*, 2004). Chronic exposure similarly blunts eCB-LTD but plasticity of the synapse is rescued by group-II mGlu receptors (Mato *et al*, 2005), suggesting that CB1R-dependent plasticity mechanisms may be replaced by alternative signaling cascades. Following extinction training from cocaine self-administration, 2-AG concentration is greatly increased in the NAc (Bystrowska *et al*, 2014). This increase in 2-AG may be compensatory for the increases in glutamatergic signalling normally seen following drug withdrawal.

While drugs of abuse seem to have limited immediate effect on CB1R control of synaptic transmission, there is evidence tying NAc eCB signaling to hedonic feeding and motivated behaviors. Notably, *Cnr1*$^{-/-}$ null mice exhibit phenotypes that coincide with reduced motivation to obtain hedonic stimuli. This is somewhat unsurprising given the known effects of ingesting *Cannabis sativa* (Stice *et al*, 2013). In rodents, 2-AG and anandamide concentrations are increased within the NAc following fasting, and intra-NAc administration of 2-AG drives voracious feeding behavior in sated rats (Kirkham *et al*, 2002). Additionally, long-term exposure to a palatable diet decreases CB1R availability in the NAc (Harrold *et al*, 2002). It is worth noting that Oginsky *et al* observed an increase in CP-AMPA expression following acute removal from palatable chow while the animals examined by Harrold *et al* were still on the diet. Taken together, it's possible that eCB signaling within the NAc is functioning reactively to changes in glutamate transmission rather than acting as a driving force for synaptic remodeling in and of itself. Future studies should focus on examining expression and function of eCB synthetic and degradative enzymes following salient

challenge.

Conclusion

While there is an ever increasing body of knowledge describing the synaptic machinery within the NAc, how synaptic plasticity influences MSN recruitment to direct neuronal circuit function remains a lofty goal for physiologists and behaviorists alike. It is also worth noting that many secondary signaling proteins recruited by the plasticity mechanisms described above are well characterized in other brain regions but have not been validated within the NAc. Given the heterogeneity of plasticity mechanisms available to individual synaptic connections within the NAc itself, these signaling cascades likewise may be unique to the NAc, differ between cell type and projecting brain region, and thus warrant additional studies.

It remains unclear how synaptic signaling mechanisms observed *ex vivo* are utilized *in vivo*, or how an animal's experiences are transduced into plasticity of accumbens synapses. It should be noted that the majority of findings summarized above pertain entirely to observations in monosynaptic connectivity and does not describe the great deal of integrative power the NAc has when considering its variety of inputs and modulatory systems function in tandem (Sesack *et al*, 2010). However, with the advent of *in vivo* imaging of neuronal activity in awake behaving animals, targeted pharmacology and optogenetics, the field is equipped to answer these questions. While these techniques have been employed to map the brain's reward circuitry, future studies should clarify how plasticity mechanisms gate synaptic function and behavior in real time. It is through these studies that we may gain insight as to how the interaction of pharmacology and physiology drive behavior in a synapse specific manner.

CHAPTER 3

mGlu1 and mGlu5 modulate distinct excitatory inputs to the nucleus accumbens shell

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Note – In accordance to the requests of the reviewers, results of statistical analyses have been placed in line with the text rather than in the figure legends.

3.1 Abstract

Glutamatergic transmission in the nucleus accumbens shell (NAcSh) is a substrate for reward learning and motivation. Metabotropic glutamate (mGlu) receptors regulate NAcSh synaptic strength by inducing long-term depression (LTD). Inputs from prefrontal cortex (PFC) and medio-dorsal thalamus (MDT) drive opposing motivated behaviors yet mGlu receptor regulation of these synapses is unexplored. Here, we examined Group I mGlu receptor regulation of PFC and MDT glutamatergic synapses onto specific populations of NAc medium spiny neurons (MSNs) using D1tdTom BAC transgenic mice and optogenetics. Synaptically-evoked long term depression (LTD) at MDT-NAcSh synapses required $mGlu_5$ but not $mGlu_1$ and was specific for D1(+) MSNs, whereas PFC LTD was expressed at both D1(+) and D1(-) MSNs and required $mGlu_1$ but not $mGlu_5$. Following five-day repeated non-contingent cocaine exposure, LTD was attenuated at MDT-D1(+) synapses but was rescued by a $mGlu_5$ positive allosteric modulator (PAM), VU0409551. These results highlight unique plasticity mechanisms regulating specific NAcSh synapses.

3.2 Introduction

The nucleus accumbens (NAc) integrates excitatory inputs encoding salient neuronal information, directing reward acquisition and shaping motivated behaviors. Strengthening discrete afferent-target glutamatergic synapses is thought to bias cellular computation of action selection by NAc circuitry and is widely regarded as a critical physiological process underlying reward learning and memory (Koob, 2011; Luscher *et al*, 2011). Repeated exposure to salient stimuli restructures these synaptic connections, redirecting future responses to stimulus presentation. Maladaptive changes in synaptic strength and synaptic plasticity are implicated in the motivational deficits observed in depression, anxiety, and drug abuse (Joffe *et al*, 2014; Russo *et al*, 2013). Understanding the molecular components underlying plasticity at specific NAc synapses may direct therapeutic interventions for treating motivational disorders.

NAc MSNs can be distinguished by their expression of either dopamine receptor type-1 [D1(+)], or type-2 [defined herein as D1(-)], and downstream projection targets (Britt *et al*, 2012; Grueter *et al*, 2012; Kreitzer and Malenka, 2008). Canonically, D1(+) MSNs promote reward seeking while D1(-) MSNs promote aversion (Calipari *et al*, 2016; Smith *et al*, 2013). Excitatory inputs onto NAc MSNs are associated with unique characteristics of motivated behavior (Everitt *et al*, 2005). Glutamatergic inputs from the prefrontal cortex (PFC) and the medial dorsal thalamus (MDT) have contrasting effects on behavioral output. Animals will self-stimulate PFC-NAc afferents and activation promotes real-time place preference (Britt *et al*, 2012). Activation of MDT afferents inhibits acquisition of palatable rewards while inhibition alleviates negative affective behaviors associated with opiate withdrawal (Do-Monte *et al*, 2017; Zhu *et al*, 2016).

Within the NAc, post-synaptic Group I mGlu receptors, comprised of $mGlu_1$ and $mGlu_5$, are known to trigger LTD of excitatory synaptic transmission. Activation of NAc group I mGlu

receptors induces a LTD of excitatory post-synaptic currents (EPSCs) via retrograde endocannabinoid signaling and/or post-synaptic internalization of AMPA receptors (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; Loweth *et al*, 2014a; Loweth *et al*, 2014b; McCutcheon *et al*, 2011a; Ohno-Shosaku *et al*, 2014; Robbe *et al*, 2002). Notably NAc $mGlu_5$ function is blunted/absent in mice exposed to cocaine via reduced surface expression (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; Huang *et al*, 2011a; Szumlinski *et al*, 2006). Augmenting mGlu function using positive allosteric modulators (PAMs), agonists, or antagonists can inhibit drug-seeking behaviors and drug-induced physiological changes in mice (Grueter *et al*, 2008; Grueter *et al*, 2007; Loweth *et al*, 2013, 2014b). However, remodeling of NAc circuits following drug exposure differs by subregion, cell type, and afferent origin (Britt *et al*, 2012; Grueter *et al*, 2012; Stuber *et al*, 2012). Elucidating how mGlu signaling modifies excitatory drive at discrete synapses is critical for understanding how they regulate the propagation of information through reward circuits.

Here, we utilize whole-cell patch clamp electrophysiology in *D1tdTom* BAC transgenic mice, viral-mediated gene transfer of channel rhodopsin (ChR2), and pharmacology to define modulation of PFC and MDT synapses onto NAcSh MSNs by mGlu receptors. We find low-frequency stimulation (LFS) of ChR2+ terminals elicits $mGlu_1$ and $mGlu_5$ dependent long-term depression (LTD) that is defined by afferent origin. Additionally, prior cocaine exposure inhibits $mGlu_5$ dependent LTD at MDT-D1(+) synapses which is rescued by application of an $mGlu_5$ positive allosteric modulator (PAM). Together, our results suggest that Group I mGlu receptor signaling selectively regulates distinct synaptic connections in the NAc of drug-naïve animals and is impaired by cocaine history in a synapse specific manner. These findings also suggest targeting of $mGlu_5$ may be valuable in treating nuanced maladaptive synaptic remodeling following substance abuse.

3.3 Results

PFC and MDT innervate NAc MSNs

Both cortical (PFC) and thalamic (MDT) inputs into the NAc form excitatory synapses onto NAc MSNs (Joffe *et al*, 2016; Ma *et al*, 2014; Neumann *et al*, 2016; Pascoli *et al*, 2011; Terrier *et al*, 2016; Zhu *et al*, 2016). Whole-cell patch-clamp electrophysiology was performed on acute parasagittal brain slices of *d1tdTom* BAC transgenic mice in order to isolate these NAcSh excitatory connections in a cell-type specific manner. We used ChR2 to drive the activity of PFC and MDT projections in the NAcSh and uncover mechanisms mediating synaptic plasticity. AAV-CaMKII-CHR2-EYFP was stereotaxically injected into the infralimbic PFC and MDT (**Fig 3.1A**). Three weeks post infection was sufficient for robust expression of ChR2-EYFP in both cell soma and afferents within NAcSh (**Fig 3.1B**). A brief (0.5-1 ms) pulse of blue light was sufficient to activate ChR2 in EFYP+ neurons and evoke robust excitatory currents and action potential firing with high fidelity (**Fig 3.1C**).

In the NAcSh, light-evoked EPSCs were observed at PFC and MDT synapses onto both MSN subtypes. EPSC amplitude varied with stimulation intensity, duration, and efficiency of viral infection at the injection site; however, values were not significantly different based on the synapse sampled (**Fig 3.1D,E**). Interestingly, EPSC waveforms exhibited significant differences in decay kinetics of AMPA currents across cell types and inputs. (**Fig 3.1F**). Specifically, -70 $t_{1/2}$ was significantly greater at MDT synapses compared to those from the PFC of the same cell type [PFC-D1(+) vs MDT-D1(+), $p<0.01$; PFC-D1(-) vs MDT-D1(-), $p<0.05$, one way ANOVA, Tukey's multiple comparisons test]. These findings demonstrated both PFC and MDT form strong excitatory connections with NAcSh MSNs.

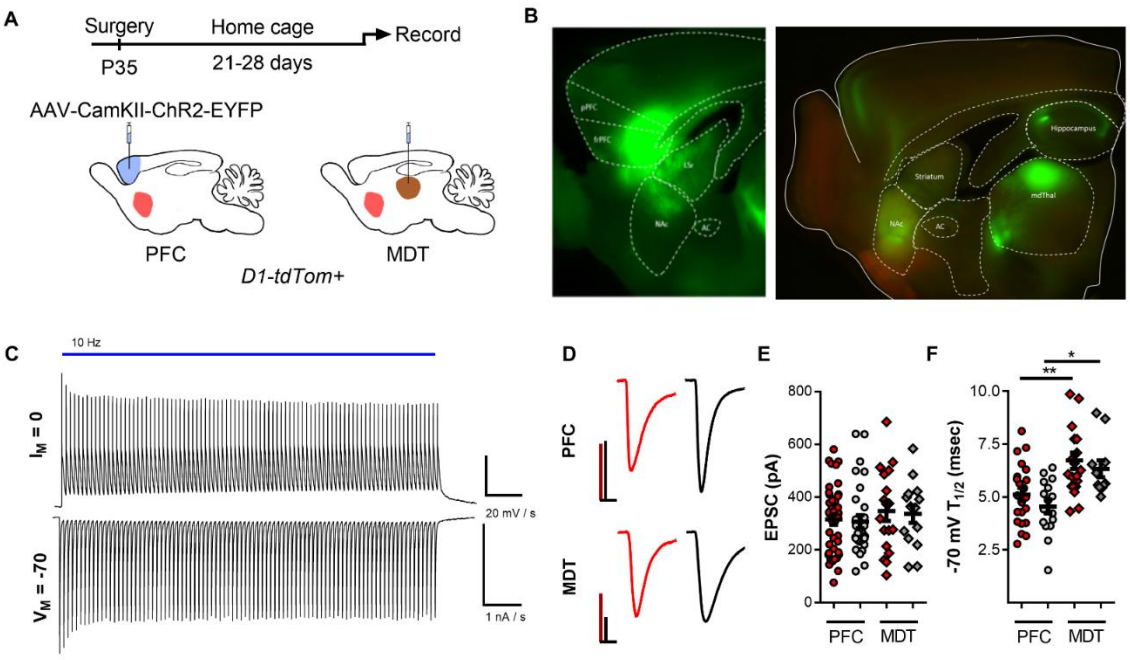


Fig 3.1 - AAV-CamKII-ChR2-EYFP infection in the PFC and MDT results in robust expression of ChR2 at injection site and NAc terminals. **A.** Experimental timeline for recording optically evoked EPSCs in the NAc. **B.** Widefield fluorescence imaging of EYFP expression in the PFC, MDT, and resulting expression in NAc afferent fibers. **C.** Representative optically evoked action-potential firing [top] and positive current [bottom] via 10 Hz stimulation of ChR2 in an EYFP expressing neuron. **D.** Representative optically-evoked EPSCs elicited by light stimulation of PFC [top] and MDT [bottom] synapses onto NAc D1(+) [red] and D1(-) [black] MSNs. Scale bars: 100pA/10ms. **E.** Summary plot of optical EPSC amplitudes recorded from NAc MSNs in all naïve LTD experiments prior to LFS. **F.** Decay kinetics for optical EPSCs evoked from PFC and MDT synapses in the NAcSh

mGlu receptor-dependent LTD is differentially expressed at PFC and MDT synapses

Recent work has demonstrated that inputs into the NAcSh are susceptible to differing types of synaptic modulation based on afferent origin (Pascoli *et al*, 2014). Several studies have demonstrated that 10-13 Hz stimulation of NAc core glutamatergic afferents induces an $mGlu_5$-dependent LTD (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; Robbe *et al*, 2002). Therefore, we sought to determine whether this mGlu-dependent LTD was similarly induced in the NAcSh adding both cell type and input specificity. Following a five minute 10 Hz stimulation, we observed a robust light-evoked LTD at PFC synapses at both D1(+) and D1(-) MSNs (**Fig 3.2A-D** PFC-D1(+), 69.16 ± 5.65, n=10, $p<0.01$; PFC-D1(-), 72.05 ± 4.327, n=10, $p<0.001$ one sample t-test). However, LFS induced LTD at MDT synapses onto D1(+) but not D1(-) MSNs (**Fig 3.2F-I** MDT-D1(+), 75.76 ± 6.221, n=10, $p<0.01$; MDT-D1(-), 104.3 ± 7.487, n=7, p=0.584, one sample t-test). Surprisingly, we were not able to reliably sample the paired pulse ratio (PPR) at PFC and MDT synapses, as paired stimulation resulted in run-down and near failures following repeated exposure (data not shown). In lieu of this, $1/CV^2$ was analyzed. Light-evoked LTD did not induce a change in $1/CV^2$ at PFC or MDT synapses (**Fig 3.2E,J** PFC: D1(+) Base vs. LTD, p=0.271; D1(-) Base vs. LTD, p=0.39, paired t-test. MDT: D1(+) Base vs. LTD, p=0.893; D1(-) Base vs. LTD, p=0.149, paired t-test). These results suggested that this mechanism likely does not involve presynaptic changes. We concluded that LTD is present at PFC synapses onto both D1(+) and D1(-) NAcSh MSNs while LTD is specific for MDT-D1(+) synapses and likely occurs via a post-synaptic mechanism.

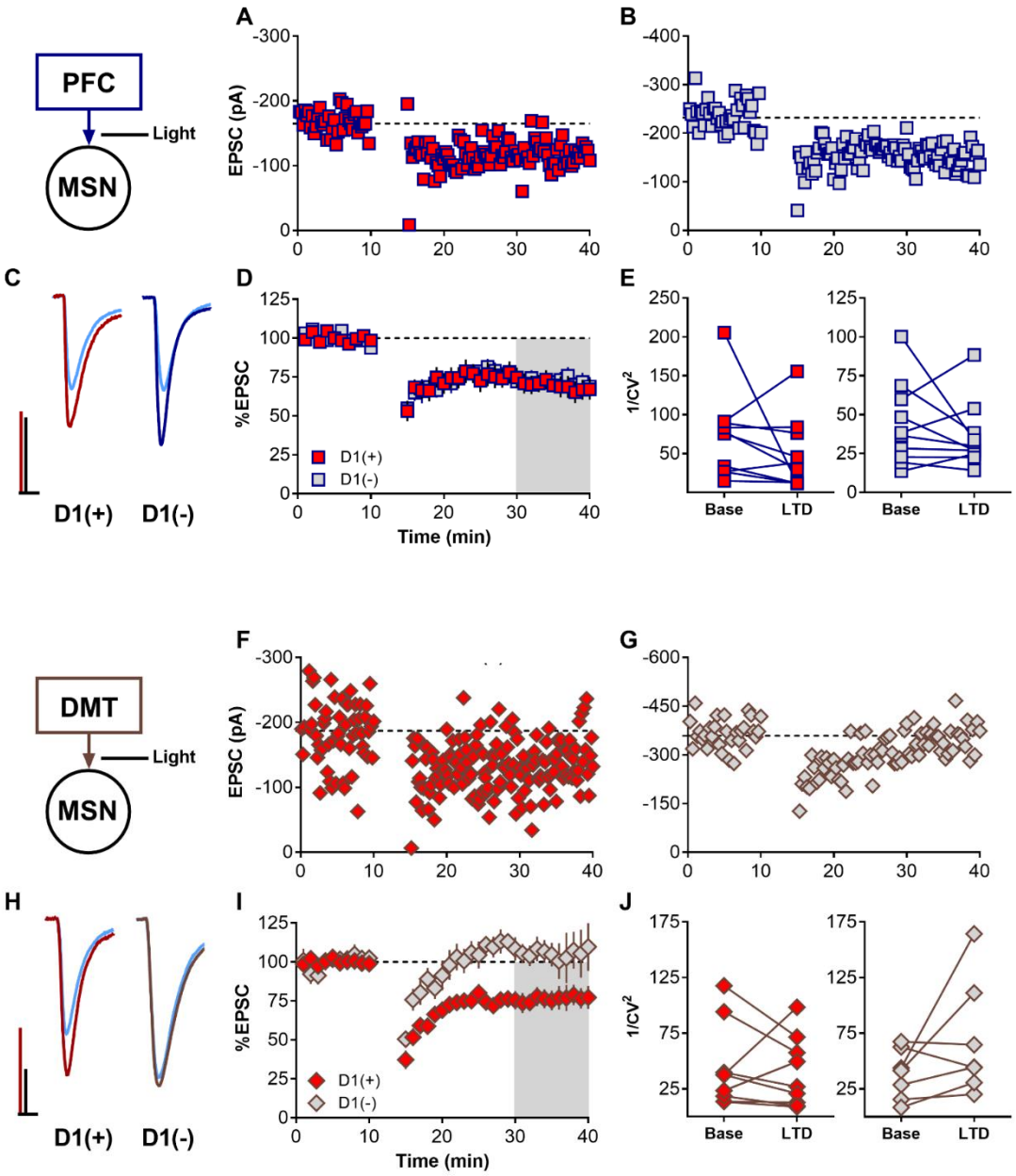


Fig 3.2 - LFS of PFC and MDT inputs to NAcSh elicits robust LTD. **A,B.** Representative LTD induction at PFC D1(+) [red] and D1(-) [gray] synapses. **C.** Representative traces averaged from baseline and the last ten minutes following LTD induction (30-40). Scale bars 100 pA/10 ms. **D**. Averaged LTD experiments at PFC synapses. E 1/CV2 during baseline and following LTD induction. **F,G.** Representative LTD induction at MDT D1(+) and D1(-) synapses. **H.** Representative traces averaged from baseline and the last ten minutes following LTD induction (min 30-40). Scale bars 100 pA/10 ms. **I.** Averaged LTD experiments sampled at MDT synapses. LTD was not evoked at MDT D1(-) synapses but was present at D1(+). **J.** 1/CV2 during baseline and following LTD induction.

To determine the molecular mediators of 10 Hz LTD at PFC and MDT synapses in the NAcSh, we utilized pharmacological antagonists of Group I mGlu receptors. We found that 10 Hz LTD was intact in the presence of the $mGlu_5$ antagonist MPEP at both D1(+) and D1(-) PFC synapses (**Fig 3.3A,C** D1(+) + MPEP, 69.07 ± 4.962, n=6, p>0.05; D1(-) + MPEP, 58.0 ± 12.6, n=5, p>0.05, one sample t-test vs. 100; D1(+) Naïve vs. MPEP, p>0.05; D1(-) Naïve vs. MPEP, p>0.05, t-test). As LTD was not observed at MDT D1(-) synapses we focused on MDT-D1(+). However, LTD was blocked by MPEP at MDT-D1(+) synapses (**Fig 3.3B,C** D1(+) 103.7 ± 7.651, p=0.652, n=6, one sample t-test vs. 100; Naïve vs. MPEP, p<0.01, t-test), suggesting $mGlu_5$ regulation of glutamatergic transmission is synapse specific. $mGlu_1$ has been shown to regulate PFC afferents in the NAcSh but only in rats withdrawn from cocaine self-administration (Ma *et al*, 2014; McCutcheon *et al*, 2011a; McCutcheon *et al*, 2011b). Nevertheless, we examined whether 10 Hz stimulation of PFC-NAc synapses was dependent on $mGlu_1$ activation. Surprisingly, we found that the $mGlu_1$ antagonist LY367385 (50 μM) was indeed able to completely block LTD at PFC-NAcSh synapses (**Fig 3.3D,F** D1(+) + LY, 106.9 ± 10.78, n=7, p=0.548; D1(-) + LY, 98.84 ± 13.4, n=5, p=0.934, one sample t-test vs. 100; D1(+) Naïve vs. LY, p<0.01, D1(-) Naïve vs. LY, p<0.05, t-test). The presence of 50 μM LY367385 did not block but enhanced LTD at MDT-D1(+) synapses (**Fig 3.3E,F** D1(+) + LY, 24.8 ± 2.404, n=6, p<0.001, one sample t-test vs. 100; D1(+) Naïve vs LY, p<0.01, t-test). These results demonstrated that $mGlu_1$-mediated LTD is present at PFC-NAcSh synapses while LTD at MDT-D1(+) MSNs requires $mGlu_5$ in naïve mice.

In order to verify that Group I mGlu receptor activation was sufficient to induce LTD we applied the Group I mGlu agonist DHPG. 100 μM DHPG induced a robust depression of synaptic transmission at PFC and MDT synapses onto NAC MSNs (**Fig 3.3G-I** PFC: D1(+)+DHPG, 63.51

± 9.523, n=7, $p<0.01$; D1(-)+DHPG, 66.66 ± 11.5, n=6, $p<0.05$, one sample t-test vs. 100. MDT: D1(+)+DHPG, 74.52 ± 6.97, n=7, $p<0.05$, one sample t-test vs. 100). We concluded that mGlu receptors function differentially at PFC and MDT NAc synapses to regulate synaptic strength.

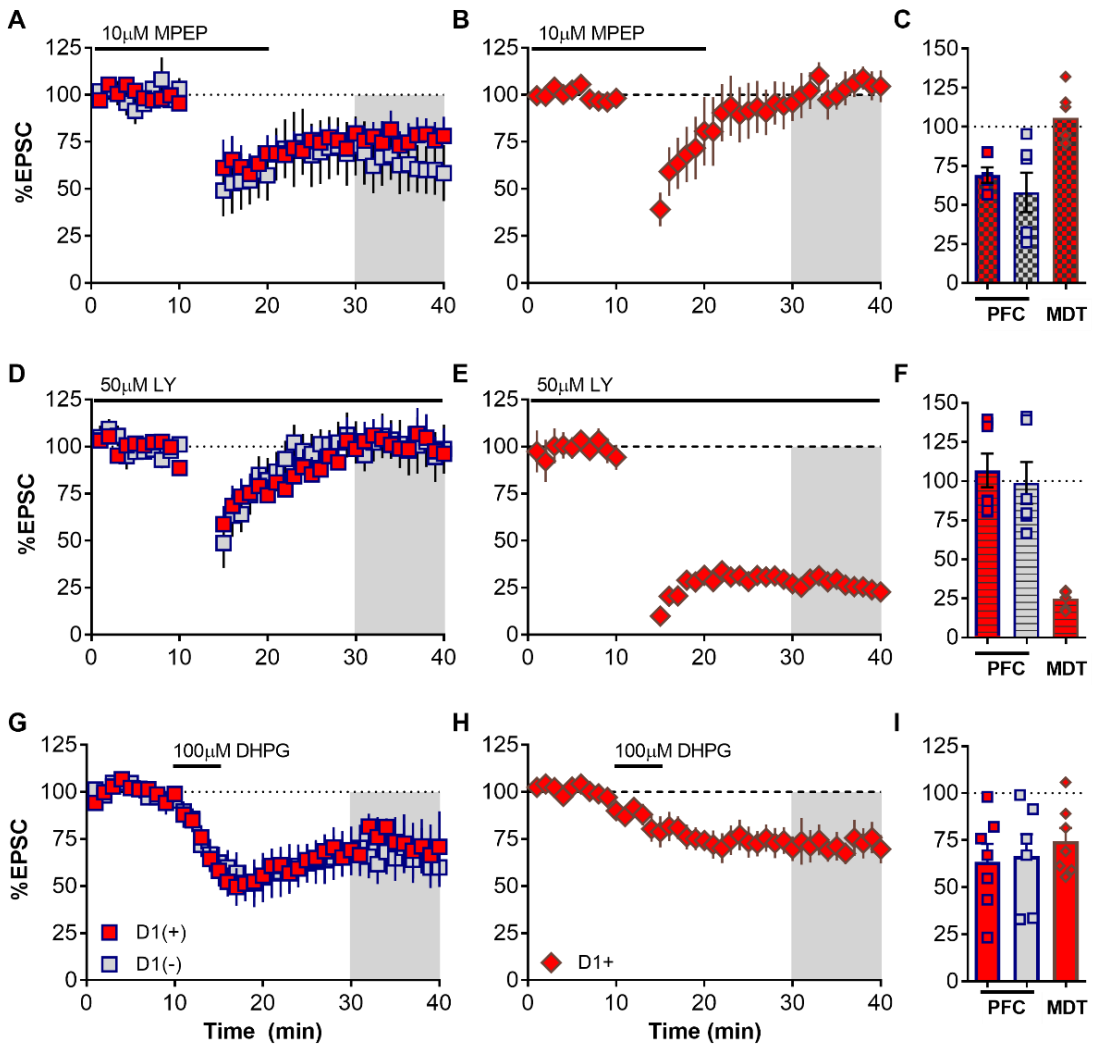


Fig 3.3 - LTD of PFC and MDT synapses is differentially controlled by Group I mGlu receptor subtype. **A.** Bath application of 10 μM MPEP does not impair LTD of PFC-NAcSh synapses. **B.** Bath application of MPEP completely blocks induction of LTD at MDT- D1(+) NAcSh synapses. **C.** Summary of experiments performed in the presence of 10 μM MPEP. Values shown as percent baseline. **D.** Application of 50 μM LY367385 completely blocks induction of LTD at PFC-NAcSh synapses. **E.** LY367385 did not impair LTD at MDT-D1(+) synapses. **F.** Summary of experiments performed in the presence of 50 μM LY367385. Values shown as percent baseline. **G,H.** Five minute bath application of the Group I mGlu agonist R,S-DHPG (100 μM) induces a robust LTD at both PFC and MDT-D1(+)synapses in the NAcSh. **I**. Summary of DHPG experiments at PFC and MDT synapses. Values shown as percent baseline.

Cocaine selectively impairs mGlu-LTD at D1(+) MSNs

Impairments in mGlu function in the NAc have been correlated to both acute and repeated exposure to cocaine. Diminished pharmacological mGlu LTD has been observed in the NAcSh 14-21 day drug-free period, which we refer to herein as "abstinence," from repeated cocaine exposure (Huang *et al*, 2011a). Therefore, we chose to examine whether a repeated drug-exposure and abstinence paradigm would impair mGlu LTD at PFC and MDT synapses. Following stereotaxic surgery, mice were subjected to a seven day cocaine sensitization task (**Fig 3.4A**) before being returned to the home-cage for a 14 day abstinence. Across repeated cocaine injections, mice exhibited a robust increase in locomotor activity relative to saline treated controls and relative to the first day of cocaine exposure, consistent with drug induced neurological adaptations (**Fig 3.4A** – Two way ANOVA of Saline vs Cocaine, interaction $F(6, 66) = 18.1$. *** $p<0.001$, **** $p<0.0001$, Cocaine compared to day one of drug, Dunnet's multiple comparisons. # $p<0.05$, ### $p<0.01$, #### $p<0.001$, saline vs. cocaine (daily), Sidak's multiple comparisons). Following cocaine abstinence, we observed a robust LTD at PFC-D1(-) synapses in both saline and cocaine treated mice (**Fig 3.4C** Saline, 67.45 ± 7.658, $n=5$, $p<0.05$; cocaine, 66.93 ± 5.329, $n=7$, $p<0.05$, one sample t-test). Similarly, LFS-LTD at PFC-D1(+) synapses was not significantly inhibited in mice exposed to cocaine when compared to saline controls (**Fig 3.4B,C** PFC-D1(+), saline, 72.83 ± 6.60, $n=11$, $p<0.05$; cocaine, 87.83 ± 3.581, $n=9$, $p<0.05$, one sample t-test. Sal vs. Coc, $p=0.07$, t-test). However, we observed a trend towards a reduction in LTD magnitude, indicating LTD may be impaired but not absent. Interestingly, LFS-LTD of MDT synapses onto D1(+) synapses was absent in mice treated with cocaine (**Fig 3.4E,G** saline, 68.1 ± 6.38, $n=5$, $p<0.01$; cocaine, 95.32 ± 6.24, $n=6$, $p>0.05$, one sample t-test. Sal vs. Coc, $p<0.05$, t-test). LFS-LTD was still absent at MDT-D1(-) synapses in cocaine treated mice (MDT-D1(-) + Coc, 108.6 ± 19.9, $n=3$, data not

shown). Taken together, these results suggested that $mGlu_5$ dependent LFS-LTD is selectively impaired MDT-D1(+) synapses in mice exposed to cocaine.

The $mGlu_5$ positive allosteric modulator VU0409551 rescues MDT LTD impaired by cocaine experience

Modulation of Group I mGlu receptors has been proposed as a valuable therapeutic strategy for treating a range of psychiatric disorders. Recent findings have demonstrated that $mGlu_5$ PAMs have strong efficacy in ameliorating behavioral deficits in a schizophrenia mouse model (Foster and Conn, 2017). Additionally, $mGlu_1$ PAMs have been efficacious in reducing cue-induced drug seeking in rats and reverse some aspects of physiological adaptations in the NAc (McCutcheon *et al*, 2011a). Therefore, having demonstrated that an $mGlu_5$-dependent LTD at MDT-D1(+) synapses is impaired following cocaine, we chose to examine whether a selective $mGlu_5$ PAM, VU0409551 (VU551), was sufficient rescue LTD in cocaine treated mice. To assess this, 10 uM VU551 (PAM) was included in the ACSF during baseline and LTD induction. LTD at MDT-D1(+) synapses was rescued in the presence of VU551 to levels near saline controls (**Fig 3.4F,G**). These results demonstrate that potentiating $mGlu_5$ can restore plasticity at MDT-D1(+) NAcSh synapses.

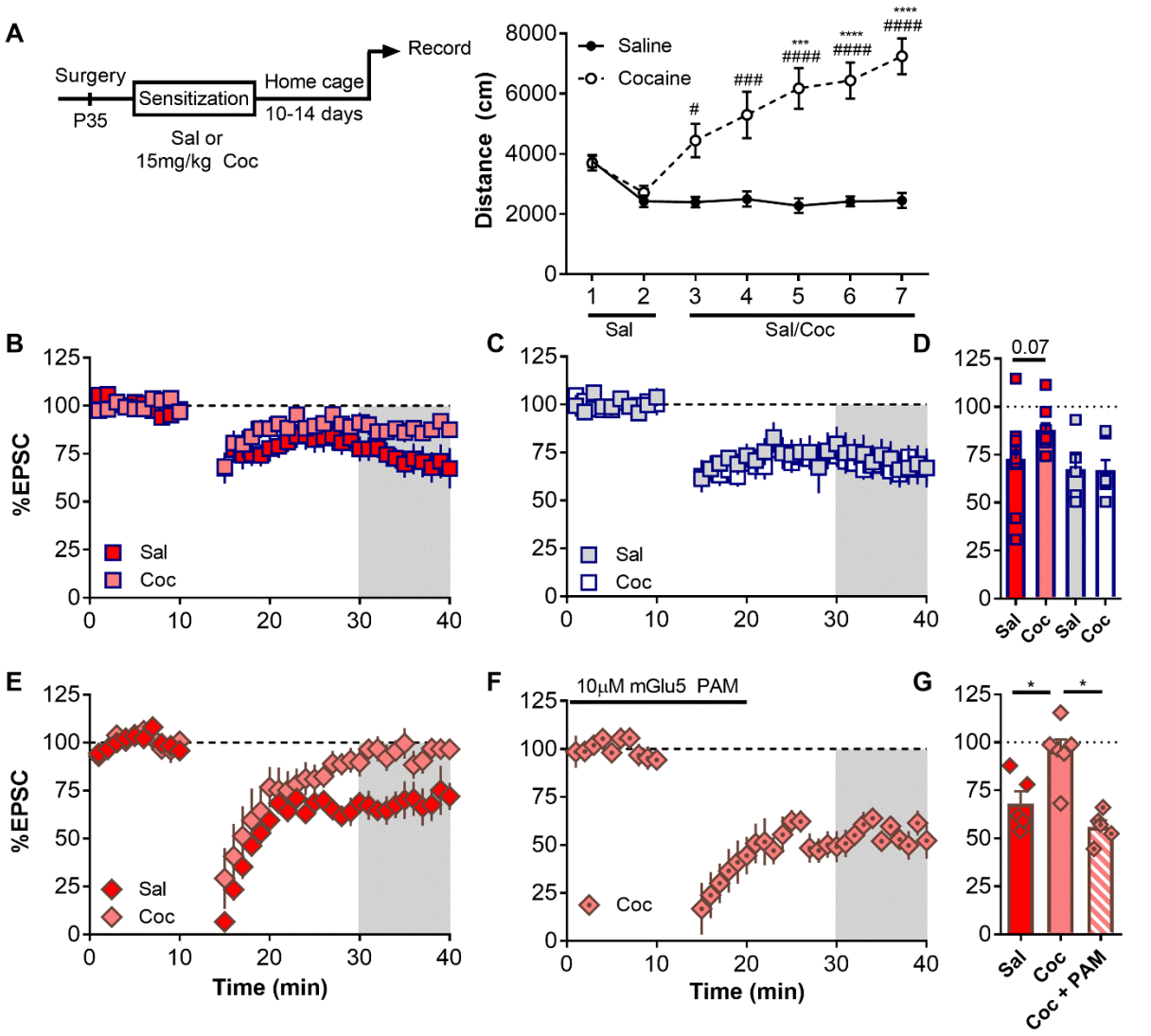


Fig 3.4 - LTD at D1(+) synapses in the NAc is impaired following cocaine exposure. **A.** Timeline of experimental procedures leading up to electrophysiological recordings. Mice exposed to cocaine (15mg/kg) exhibited robust locomotor sensitization following repeated exposures. Representative motion capture traces of mice on day two of saline (1) and final cocaine dosing (2). **B.** LTD at PFC D1(+) MSNs was not significantly impaired in mice exposed to cocaine compared to saline controls and EPSC amplitude was significantly different from baseline in cocaine-LTD experiments. **C.** LTD at PFC D1(-) MSNs was not affected by cocaine exposure. **D.** Summary of LTD experiments from sal/coc exposed mice at PFC-NAc synapses. Values shown as percent baseline. **E.** LTD at MDT D1(+) synapses was absent in mice exposed to cocaine and significantly different from saline controls. **F.** Bath application of the $mGlu_5$ PAM VU551 (10 μM) was sufficient to rescue LTD deficits seen at MDT-D1(+) synapses in mice exposed to cocaine. **G.** Summary of LTD experiments from sal/coc exposed mice at MDT D1(+) synapses. Values shown as percent baseline.

3.4 Discussion

NAc mGlu receptors are potent regulators of glutamatergic synaptic strength (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; Robbe *et al*, 2002; Turner *et al*, 2018) and are impacted by cocaine history (Grueter *et al*, 2007; Wolf, 2016). However, how these receptors control excitatory transmission from discrete inputs is unknown. Here, we demonstrated an $mGlu_1$ LFS-LTD is present at PFC synapses onto both cell types, but plasticity from MDT inputs is specific for D1(+) MSNs and requires $mGlu_5$. Following cocaine exposure, this plasticity is attenuated selectively at MDT D1(+) synapses (**Fig 3.5**). These results are consistent with recent studies demonstrating a heterogeneity of molecular regulatory mechanisms functioning at specific NAc synapses and input and cell-type specific changes induced by drug exposure ((Joffe *et al*, 2016; Ma *et al*, 2014; Neumann *et al*, 2016; Pascoli *et al*, 2014; Zhu *et al*, 2016). These findings broaden the known role of mGlu receptors in shaping reward circuit function.

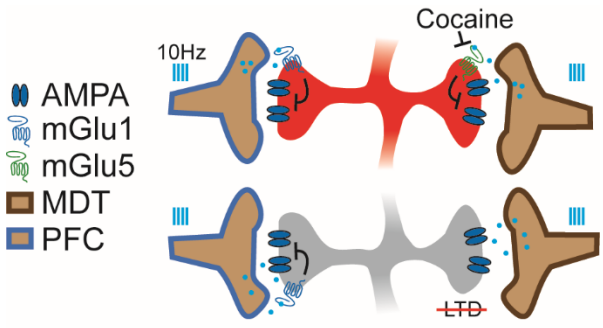


Fig 3.5 - Model of mGlu regulation of NAcSh PFC and MDT synapses. 10Hz light-evoked Group I mGlu LTD is differentially expressed at PFC and MDT synapses, with cortical synapses recruiting $mGlu_1$ while thalamic inputs onto D1(+) MSNs recruits $mGlu_5$. LTD at all synapses likely occurs via a post-synaptic mechanism as indicated by changes in $1/CV^2$. Prior cocaine exposure blocks the expression of LTD at thalamic – D1(+) synapses but does not impair $mGlu_1$ LTD at PFC synapses.

Distinct glutamatergic NAcSh inputs are hypothesized to drive nuanced aspects of motivated behavior. Specifically, the PFC is thought to direct reward seeking and enhance positive environment-associated valence (Britt *et al*, 2012; Everitt *et al*, 2005). Conversely, inputs from the MDT have been shown to drive conditioned place aversion and inhibit palatable reward seeking (Do-Monte *et al*, 2017). Using region-specific expression of ChR2 (**Fig 3.1A,B**) and D1-tdTom marker mice, we determined that, despite these differences in behavioral affect, light-evoked EPSCs did not vary in amplitude across inputs or between cell types. However, we observed significantly greater decay kinetics at MDT-NAc synapses compared to PFC (**Fig 3.1E**). While differences in kinetics have been observed due to AMPA subunit composition (Lu *et al*, 2009), these results are inconsistent with recent findings showing the presence of GluA2-lacking AMPA receptors in naïve and saline treated rats at MDT-NAc synapses (Neumann *et al*, 2016). However, these differences could also be explained by synaptic morphology, dendritic locus, or AMPA auxiliary protein association (Greger *et al*, 2017; Herring *et al*, 2013) and may serve as the basis for future studies.

We next determined whether mGlu receptor-dependent LTD occurs at PFC and MDT synapses. We found that optical LFS is able to induce a robust LTD at PFC synapses on both MSN subtypes (**Fig 3.2**). However, LTD at MDT synapses was specific for D1(+) MSNs. Additionally, light-evoked LTD was not accompanied by a change in $1/CV^2$ (**Fig. 3.2H,I**) suggesting it may occur-via a post-synaptic mechanism. We next confirmed the necessity of Group I mGlu receptor activation for light-evoked LTD at PFC and MDT NAcSh synapses. Using specific Group I mGlu receptor antagonists, we found that LTD at PFC-NAcSh synapses was independent of $mGlu_5$ but required $mGlu_1$ (**Fig 3.3**). This result was surprising as previous publications have almost exclusively implicated $mGlu_5$ as the triggering mechanism for this plasticity within the NAc.

Specifically, Ma *et al* demonstrated an $mGlu_1$-dependent plasticity at PFC-NAc synapses using a 1Hz stimulation protocol that also required NMDA receptors in rats trained to self-administer cocaine. Additionally, others have shown a switch from $mGlu_5$-dependent eCB signaling in drug-naïve animals to an $mGlu_1$-dependent internalization of AMPA receptors in animals that had self-administered cocaine (Ma *et al*, 2014; McCutcheon *et al*, 2011a). However, these studies did not differentiate MSN subtype, were performed in rats, and evoked Group I mGlu LTD using a different stimulation protocol or the Group I mGlu agonist DHPG. Thus, our findings suggest that $mGlu_1$ activation may favor PFC inputs and require more substantial glutamate release for recruitment in naïve animals.

Unlike the PFC, LFS-LTD of MDT inputs was specific for D1(+) MSNs. The presence of this LTD specifically at D1(+) MSNs may contribute to the aversive effects of 10-30Hz stimulation of MDT synapses in the NAc *in vivo* (Do-Monte *et al*, 2017). LTD of D1(+) but not D1(-) would bias MDT-driven NAc function towards D1(-) MSNs output, a phenomenon shown to oppose appetitive behavior (Lobo *et al*, 2010). Additionally, this plasticity was blocked by the $mGlu_5$ antagonist MPEP and insensitive to the $mGlu_1$ antagonist LY367385 (**Fig 3.5D,E**). Bath application of DHPG was similarly sufficient to induce LTD at MDT-D1(+) synapses. Taken together, these findings demonstrate an input and cell-type specific regulation of NAc synapses by Group I mGlu receptors, with pan Group I activation favoring decreased PFC influence and enhanced MDT-D1(-) excitatory drive. While this demonstrated differential regulation of these inputs by Group I mGlu receptors, both $mGlu_1$ and $mGlu_5$ are expressed at PFC and MDT NAc terminals (Mitrano *et al*, 2010). Thus, both $mGlu_1$ and $mGlu_5$ are likely functional but are recruited by different conditions.

Alterations in glutamatergic transmission in the NAcSh occur following

abstinence/withdrawal from cocaine, a physiological correlate of the incubation of drug craving (Fourgeaud *et al*, 2004; Wolf, 2016). These changes broadly include an increase in glutamatergic quantal size, changes in MSN excitability, generation of silent synapses, increases in presynaptic release, and disruptions in mGlu-dependent plasticity (Grueter *et al*, 2012). Thus, we investigated whether mGlu-dependent LTD was affected following abstinence from cocaine at PFC and MDT synapses. We observed no significant difference in LFS-LTD at PFC synapses in mice exposed to cocaine compared to saline controls (**Fig 3.4C,D**). However, LFS-LTD at MDT-D1(+) synapses was absent in mice exposed to cocaine. This is consistent with multiple reports demonstrating cocaine-induced adaptations in NAc circuitry is specific for D1(+) MSNs (MacAskill *et al*, 2014; Pascoli *et al*, 2014; Terrier *et al*, 2016) as well as observed deficits in mGlu-dependent plasticity in rodents following cocaine (Fourgeaud *et al*, 2004; Grueter *et al*, 2010; McCutcheon *et al*, 2011a). These findings are supported by decreased DHPG-induced LTD in the NAcSh following abstinence from experimenter delivered cocaine (Huang *et al*, 2015; Huang *et al*, 2011a). While others have demonstrated enhanced LFS-LTD at PFC-D1(+) synapses following withdrawal from contingent cocaine self-administration (Pascoli *et al*, 2014), multiple reports have failed to see differences in glutamatergic synaptic strength of PFC synapses in the NAcSh following non-contingent drug delivery (Britt *et al*, 2012; Joffe *et al*, 2016). Taken together, these results are congruent with reports highlighting differential effects of contingent and experimenter-delivered drug regimens on NAc circuitry function (McCutcheon *et al*, 2011b) and attenuation of mGlu-dependent plasticity in withdrawal from non-contingent cocaine exposure.

Resetting synaptic signaling via *in vivo* optogenetics is sufficient to ameliorate drug induced behavioral adaptations. By 'normalizing' the connectivity of the PFC with the NAcSh using *in vivo* optogenetic LFS mimicking mGlu plasticity *ex vivo*, cue-induced cocaine seeking is

reduced (Pascoli *et al*, 2014). Additionally, dampening MDT input in to the NAc using hM4Di (inhibitory) designer receptors exclusively activated by designer drugs (DREADDs) or optogenetic silencing was sufficient to reduce morphine withdrawal-induced aversion (Zhu *et al*, 2016). While $mGlu_1$ agonists and PAMs have been shown efficacious in rescuing cocaine-induced physiological and behavioral effects (McCutcheon *et al*, 2011a), it is unknown whether $mGlu_5$ PAMs are also able to ameliorate drug-induced changes in the NAc. Thus, we utilized VU551, an $mGlu_5$ PAM shown to potentiate $mGlu_5$ signaling independent of NMDA receptor activation (Rook *et al*, 2015) and reduce psycho-mimetic behavior in rodents. VU551 was able to rescue the induction of $mGlu_5$ LTD at MDT-D1(+) synapses in cocaine treated mice. Notably, $mGlu_5$ in the NAc had been shown to promote resilience to chronic stress (Shin *et al*, 2015), and $mGlu_5$ antagonists are capable of reducing lever pressing for cocaine (Moussawi *et al*, 2009). We posit that the multimodal effects of targeting $mGlu_5$ may be due in part to regulation of specific afferent-MSN connections. While agonizing $mGlu_5$ appears to run counter to preventing drug seeking, targeting $mGlu_5$ may alternatively be useful for treating anhedonia, anxiety, and dysphoria following the cessation of drug intake.

Our findings demonstrate that synaptic recruitment of Group I mGlu receptors occurs differentially at discrete NAc afferents, highlighting unique roles for $mGlu_1$ and $mGlu_5$ in regulating PFC- and MDT-NAc synapses, respectively. While it is unclear whether these findings are specific to PFC and MDT synapses, these results highlight the necessity for synapse-specific approaches in future studies aimed at deconstructing molecular mediators of synaptic connectivity within the reward circuitry. Expanding our understanding of synapse-specific plasticity mechanisms serves to clarify how unique synaptic profiles allow for integration of neuronal reward-encoding information and opens the door for targeted pharmacological approaches to

remodel reward circuit function. Our results broaden the understanding of mGlu regulation of NAc reward circuitry, bolstering the potential for targeting mGlu receptors in motivational disorders.

3.5 Methods

Animals – All animals were bred and housed at Vanderbilt under the supervision of the Department of Animal Care. Transgenic BAC *Drd1a-tdTomato* mice were obtained from JAX laboratories and bred to C57BL/6J wild type females. Animals were housed on a 12-hour light/dark cycle and fed *ad lib*. Breeding cages were given access to 5LOD chow (PicoLab®, 28.7% Protein, 13.4% Fat, 57.9% Carbohydrate) to improve the viability of litters. Upon weaning at P21-28, experimental animals were switched to standard chow.

Stereotaxic surgery - All surgeries were performed in accordance to guidelines set by Vanderbilt IACUC. Briefly, 4-6 week male C57BL6 mice are anesthetized using a cocktail of ketamine (75 mg/kg) and dexdomitidor (0.5 mg/kg). Craniotomies were performed using a manual drill, AmScope microscope, and World Precision Instruments Aladdin Al-2000 syringe pump hydraulic system. Injection sites were based on coordinates listed in The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008). PFC (AP 1.4, ML ± 0.5, DV -2.9 mm) and MDT (AP -1.2, ML 0.3, DV -3.00 mm) were located using Leica AngleTwo Stereotaxic software. AAV-CamKII-ChR2-EYFP (UNC Vector Core) was injected at 100 nL per minute and allowed to permeate into the tissue for 10 minutes before removal of the syringe. Mice were revived using 0.5 mg/kg antisedan and treated with 5 mg/kg ketoprofen for three days following surgery.

Electrophysiology – Mice were anesthetized using isoflurane prior to sacrifice. Parasagittal sections (250 μm) containing the NAcSh were prepared from whole brain tissue using a Leica Vibratome. Slices were briefly placed in an N-methyl D-glucamine (NMDG) based recovery solution (2.5 KCL, 20 HEPES, 1.2 NaH_2PO_4, 25 Glucose, 93 NMDG, 30 $NaHCO_3$, 5.0 sodium

ascorbate, 3.0 sodium pyruvate, 10 $MgCl_2$, and 0.5 mM $CaCl_2$-$2H_2O$) for 10-15 min at 32°C before transfer to a chamber containing artificial cerebral spinal fluid (ACSF, 119 NaCl, 2.5 KCL, 1.3 $MgCl_2$-$6H_2O$, 2.5 $CaCl_2$-2HO, 1.0 NaH2PO4-H2O, 26.2 $NaHCO_3$, and 11 mM glucose) until use. All electrophysiology experiments were performed using a Scientifica Slicescope Pro System under a constant perfusion of 32°C ACSF at a rate of 2 mL/min. NAcSh MSNs were visualized with a Scientifica PatchVision software and patched with 3-5 MΩ recording pipettes (P1000 Micropipette Puller) filled with a cesium-based internal solution (120 $CsMeSO_3$, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4.0 Mg^2-ATP, 0.3 Na^2-GTP, 0.1 spermine, and 5.0 mM QX 314 bromide). MSNs were identified based on visual appearance (size, morphology) as well as electrophysiological properties (membrane resistance, capacitance, and the presence of currents at +40 mV to exclude fast-spiking interneurons); D1 MSNs were identified by fluorescence of tdTomato. All experiments were performed in the presence of the $GABA_A$ channel blocker picrotoxin (50 µM). Experimental protocol execution, stimulation control, and data collection were performed using a Molecular Devices pClamp 10 Analysis software. Control and monitoring of cell electrical properties were achieved using an Axopatch 500B Multiclamp amplifier and Axon Digidata 1550 low-noise data acquisition digitizer. Responses were filtered at 2 kHz and digitized at 10 kHz. Optical stimulation of ChR2-expressing terminals was achieved using a CoolLED pE-100 LED excitation system. 480 nm light was pulsed through the high-powered (40x) objective to excite ChR2+ terminals at 0.1 Hz for 0.5-1 msec. Light intensity was adjusted to evoke stable responses.

Behavior - Behavior was performed in MedAssociates Activity Test Chambers. Mice used in cocaine experiments were habituated to the behavior chambers and intraperitoneal (IP) saline injections in 15 minute sessions over two days. The following five days, mice were given a single

injection of vehicle (saline) or cocaine (15 mg/kg) IP immediately prior to being placed in the chamber. Mice were housed in home cages for the duration of the sessions and for at least two weeks following the final session before sacrifice for electrophysiology recordings. Locomotor activity was tracked using Noldus Ethovision software.

Drugs – 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP, $mGlu_5$ antagonist) , LY367385 ($mGlu_1$ antagonist), (RS)-3,5-Dihydroxyphenylglycine (DHPG, Group I mGlu agonist), ketoprofen, dexdomitodor, and antisedan, were obtained from Tocris. Picrotoxin and cocaine hydrochloride were obtained from Sigma Aldrich. Ketamine was obtained from Patterson Veterinary Supply. VU0409551 was contributed by Jerri M. Rook, Craig W. Lindsley, and P. Jeffrey Conn.

Imaging – Widefield images were taken using an AZ-100 microscope housed in the Vanderbilt Cell Imaging Shared Resource Core facility. 250 µm brain slices originally prepared for electrophysiology experiments (see above) were fixed after recording with a 4% paraformaldehyde (PFA) solution for <24 hours and stored in a 20% w/v sucrose solution until use. Images were processed using NIS Elements Viewer (Nikon) and ImageJ.

Data Analysis – Electrophysiology experiments were analyzed using using Clampfit 10.4 and Graphpad Prism v6.0. For LTD experiments, change in baseline–and CV was calculated by averaging each value in the last ten minutes of each recording and comparing to the average across baseline events. LTD was defined as a significant difference in amplitude of the last ten minutes of the recording as measured using a one sample t-test vs. 100. Paired t-tests were performed to compare changes in CV over the course of experiments. Two-tailed t-tests were used to compare drug effects at specific synapses (defined by cell type and afferent origin). -70mV decay was analyzed using a one-way ANOVA and Tukey's post test. Behavior sensitization data was

analyzed using a two-way repeated measures ANOVA with Sidak's and Dunnet's posttests to examine individual days. For all analyses, alpha was set as 0.05. **Addendum**: In all analyses, (n) value represents the number of cells. For all groups, at least three animals are represented.

CHAPTER 4

Cortical cannabinoid receptors regulate the formation of drug-environment associations

The following section is formatted in preparation for submission to *Nature Neuroscience* as a Brief Communication.

4.1 Abstract

Top-down control of limbic brain regions via cortical afferents is extensively linked to the initiation of motivated behaviors. Aberrant reorganization of cortical output can lead to the generation of unwanted behavioral paradigms including those associated with addiction. Here, we report that regulation of cortical projection neurons by cannabinoid type-1 receptors is essential to the formation of cocaine-environment association. Additionally, our results suggest that cocaine reorganizes cortical synapses in the nucleus accumbens to bias output in favor of action initiation via post-synaptic regulation of endocannabinoid production. These findings provide a potential mechanism by which forebrain cannabinoid signaling restructures neuronal circuitry in the development of drug-reward associations.

4.2 Main

Neocortical glutamatergic projection neurons exert broad top-down control over the formation of positively valued associations of conditioned to unconditioned stimuli, be it with social contexts (Rothwell, 2016), food (Gremel *et al*, 2016; Gremel and Costa, 2013; Land *et al*, 2014), or abused drugs (Britt *et al*, 2012; Pascoli *et al*, 2014; Pascoli *et al*, 2011; Turner *et al*, 2018). Synaptic control of forebrain circuits by cannabinoid type-1 receptors (CB1Rs) and production of their endogenous ligands are crucial mechanisms gating association formation and

driving pro-reward behaviors (Bellocchio *et al*, 2010; Ingebretson *et al*, 2017; Jung *et al*, 2012; Martin-Garcia *et al*, 2016; Orio *et al*, 2009). We chose to anatomically refine the role of CB1Rs in these behaviors by examining how CB1Rs expressed in prefrontal cortical (PFC) projection neurons influence the formation and expression of environment-stimulus associations using a viral knock-out approach and a cocaine conditioned place preference (CPP) assay. *Cnr1*$^{fl/fl}$*-cChR2*$^{fl/fl}$*-d1TdTom* mice were injected into with AAV-CamKII-Cre-GFP or AAV-CamKII-GFP in the PFC to knock-out CB1Rs prior to a cocaine conditioned place preference (CPP) assay (**Fig 4.1a**).

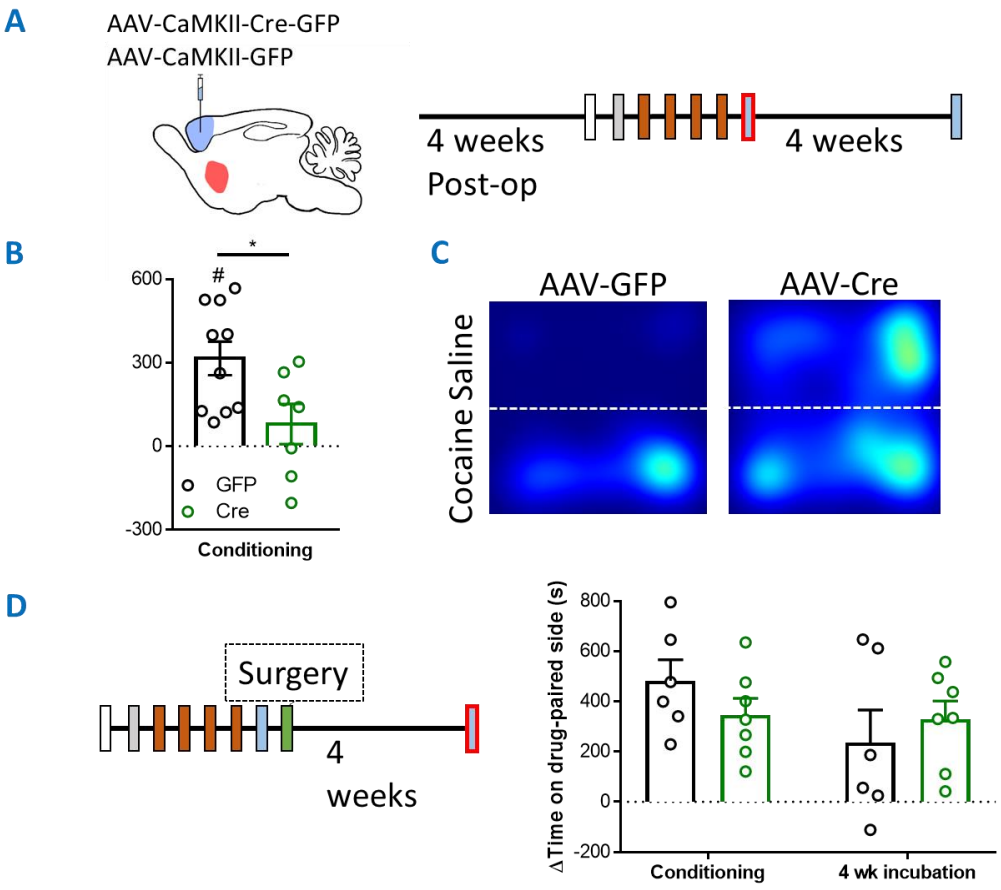


Figure 4.1 - Knockout of cortical CB1 inhibits acquisition but not expression of cocaine CPP. **A.** Schematic of experimental approach and behavior battery. **B.** Change in time on drug paired side (compared to pre-test) following a repeated conditioning sessions [2way Repeated Measures ANOVA GFP (10) vs Cre (7). Virus: $F(1,15)=9.518$, $P=0.0075$ (**); Subjects: $F(15, 15) = 2.704$, $P=0.0316$ (*). Sidak's Multiple Comparison test, GFP(10) vs Cre(7), $p<0.05$. See Supplementary Table 2 for complete statistics]. **C.** Representative heat-map images of time-spent on saline or drug paired side for GFP (left) and CRE (right) injected animals. **D.** Schematic of post-conditioning CB1 knockout approach and resulting time spent on drug paired side following a 4 week incubation period [2way ANOVA GFP (6) vs. Cre (7), n.s. See Supplementary Table 3 for complete statistics].

Four week expression of *Cre* resulted in a removal of CB1Rs from PFC axon terminals as depolarization-induced suppression of excitation evoked at PFC synapses onto nucleus accumbens (NAc) *tdTom*+ [D1(+)] or *tdTom*- [D1(-)] medium spiny neurons (MSNs) was significantly reduced compared to synapses with intact receptors and similar to experiments performed in the presence of the CB1R antagonist AM251 (**Supplementary Fig 4.1**). Mice injected with AAV-Cre failed to form a preference with the cocaine (15mg/kg IP)-paired chamber over the saline-paired side (**Fig 4.1b,c**). GFP injected mice retained their preference for the cocaine-paired chamber out to four weeks following test day while Cre-injected mice did not differ from the lack of association seen after repeated conditioning (**Supplementary Fig 4.2a,b**), demonstrating the potency of the drug-environment association formed over multiple sessions. The lack of preference for the drug paired side was not due to impairments of cortical-striatal locomotor circuitry as removal of PFC CB1Rs did not impact basal locomotor activity as assayed by a one-hour open field task performed prior to the start of CPP (**Supplementary Fig 4.2a,b**), nor did this impact anxiety-like behavior as measured by the time spent in the center of the chamber (**Supplementary Fig 4.2c**). Additionally, the lack of association was likely not due an impaired response to cocaine as mice expressing *Cre* displayed similar locomotor activity during cocaine conditioning sessions to *GFP* controls and sensitized to the drug dose across repeated sessions (**Supplementary Fig 4.2d,e**).

Having established PFC-CB1Rs are necessary for cocaine CPP acquisition, we next sought to address whether PFC-CB1Rs were necessary for maintaining the drug-environment preference. Mice were subjected to the same behavior battery but did not undergo surgery prior to initiation. Post-conditioning, animals were randomly assigned into either *Cre* or *GFP* groups and injected immediately following posttest and returned to their home cages for four weeks. Both *Cre* and *GFP* groups displayed a significant preference for the cocaine-paired side following four weeks

of viral expression at the maintenance time point (**Fig 4.1d**). These findings demonstrate that PFC-CB1Rs are necessary for the development but not the expression of cocaine CPP.

A prominent downstream projection of the PFC that may coordinate the formation of drug-environment associations is the NAc (Everitt *et al*, 2005, 2016; Goto and Grace, 2005; Ma *et al*, 2014; Turner *et al*, 2018; Volkow *et al*, 2015). Glutamatergic PFC-NAc synapses have been shown to recruit presynaptic and post-synaptic remodeling cascades following repeated cocaine self-administration (Suska *et al*, 2013), but how cocaine exposure might remodel these synapses while the drug is onboard is limited to fMRI, microdialysis, and *in vivo* amperometry approaches which cannot observe synaptic and cell-type specific changes. Therefore, we examined how *ex vivo* cocaine (10µM) delivered via ACSF to *ex vivo* slice preparations altered PFC-NAc synaptic function using whole-cell electrophysiology. AAV-CamKII-ChR2-EYFP or AAV-CaMKII-Cre-GFP were injected into *Cnr1*$^{fl/fl}$*-cChR2*$^{fl/fl}$*-d1TdTom* mice to sample PFC-NAc synapses with and without functional CB1Rs. In ChR2 injected animals, bath application of 10µM cocaine reduced EPSC amplitude evoked via optical (oEPSC) and electrical (eEPSC) stimulation (**Supplementary Fig 4.3a,b,e,f**), but this effect reversed following removal from the ACSF solution. The reduction in EPSC amplitude is consistent with observations using higher concentrations of cocaine in field potential recordings (Nicola *et al*, 1996). However, cocaine wash-on did not alter PPR of oEPSCs or eEPSCs (**Supplementary Fig 4.3c,d,g,h**). Subsequent experiments were performed at least 20 minutes following cocaine washout based on an established *ex vivo* exposure paradigm (Ingebretson *et al*, 2017). We sampled PPR at multiple inter-stimulus intervals (ISI; 20, 50, 100, 200, and 400ms) to determine if cocaine affected release probability at PFC-NAc synapses. PPR at PFC-D1(+) synapses was unchanged following *ex vivo* cocaine (**Fig 4.2a)**. However, PPR of optically-isolated PFC-D1(-) synapses was increased, indicating a reduction in release probability

(**Fig 4.2f**). This effect was likely specific as PPR of eCPSCs was unaffected (**Supplementary Fig 4.4a,b**). Additionally, DSE of oEPSCs was reduced at PFC synapses onto both D1(+) and D1(-) MSNs (**Fig 4.2b,c,g,h**). The reduction in DSE was not due to non-functionality of CB1Rs as reduction in oEPSC amplitude induced by the CB1R agonist WIN55 (1 μM) was not affected by *ex vivo* cocaine (**Supplementary Fig 4.6a-c**), nor was the WIN55 mediated reduction in electrical EPSCs impaired (**Supplementary Fig 4.6d-f**).

In order to determine if CB1Rs contribute to these synaptic effects, we performed similar experiments in mice injected with *Cre*. Removal of CB1Rs from PFC terminals did not affect release probability of eEPSCs as assessed by PPR (**Supplementary Fig 4.4**), but did prevent the cocaine induced increase in PPR at PFC-D1(-) MSNs (**Fig 4.2a,f**). Additionally, DSE of oEPSCs in *Cre* injected animals was not further impaired by *ex vivo* cocaine (**Fig 4.2d,e,I,j**) at either PFC D1(+) or D1(-) synapses. These findings demonstrate that acute exposure to cocaine modifies presynaptic release at PFC to D1(-) synapses via a CB1R-dependent mechanism and that the reduction in DSE amplitude is likely occluded by lacking CB1Rs.

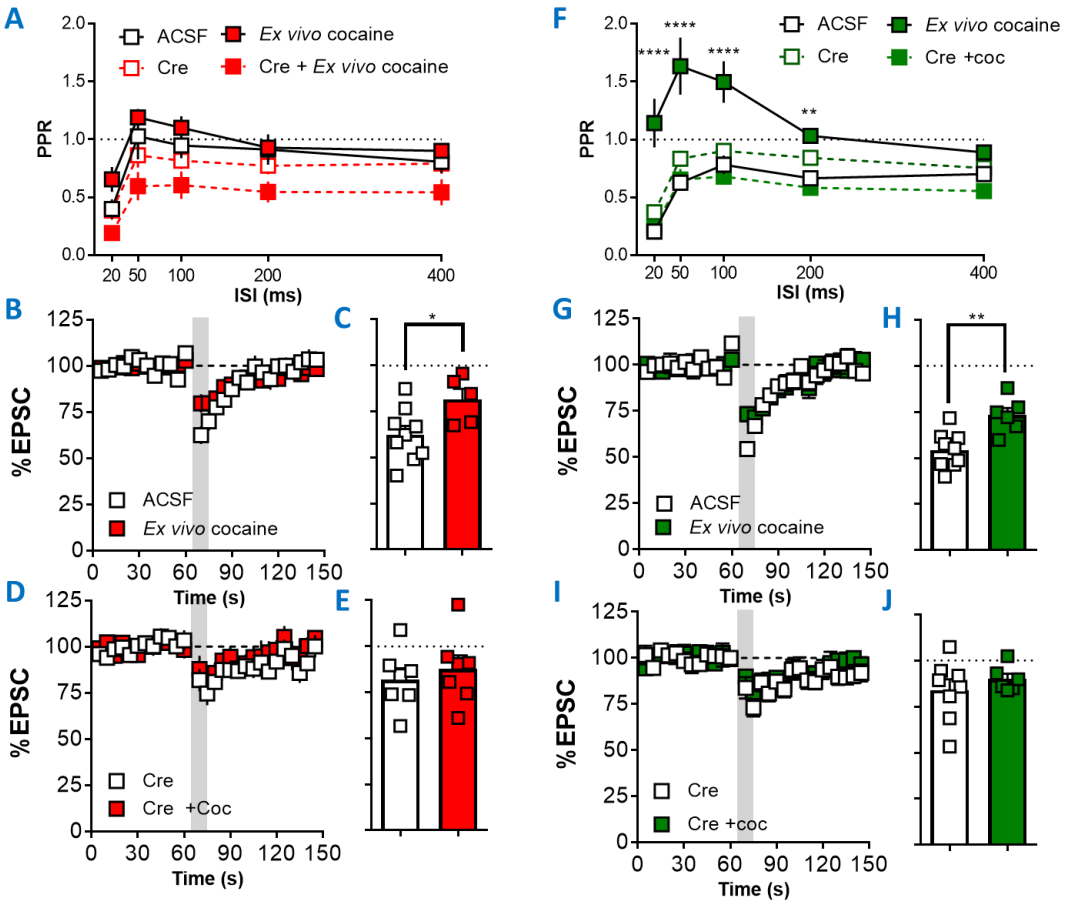
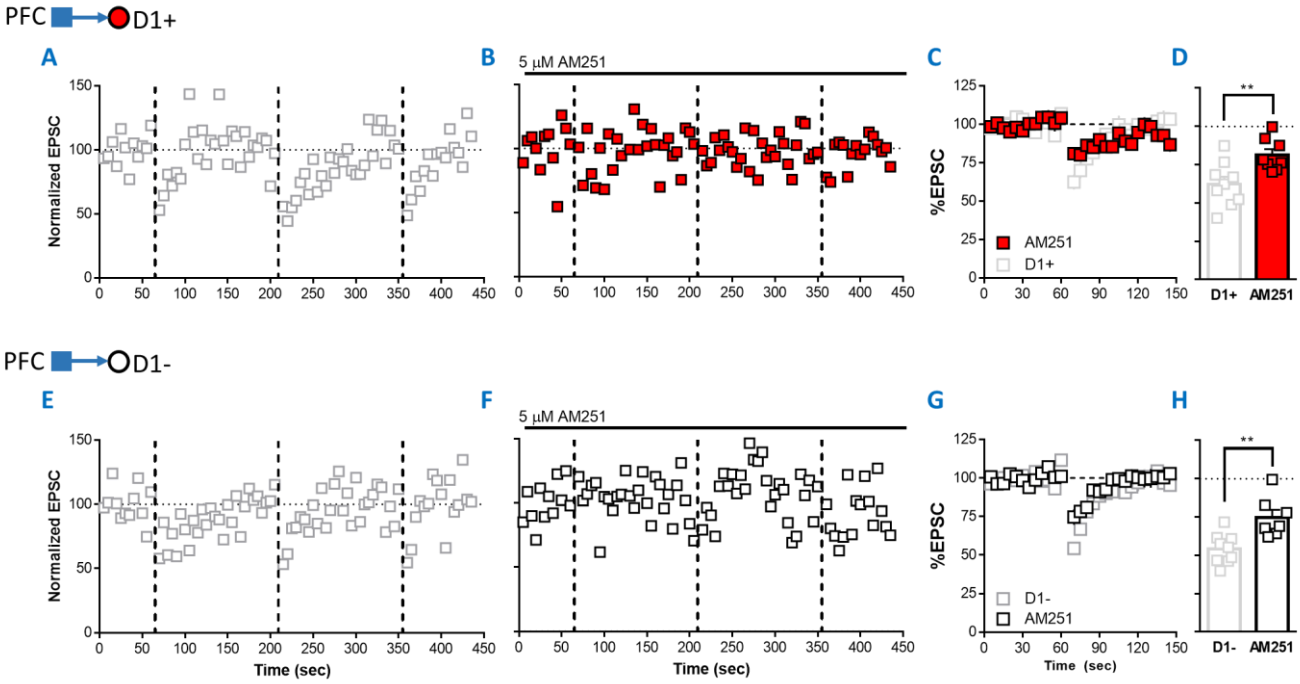


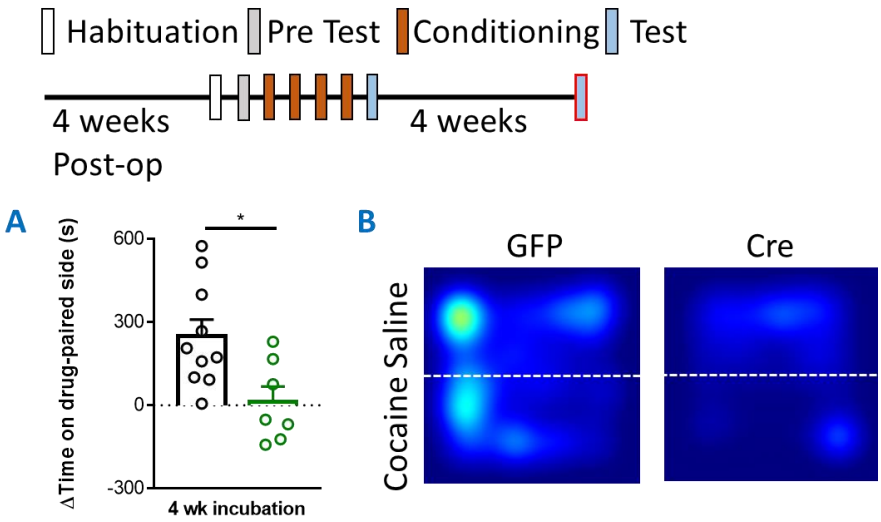
Figure 4.2 - *Ex vivo* cocaine alters cortical-accumbens presynaptic release and endocannabinoid signaling in a cell-type specific manner. **A.** Optically evoked PPR curve experiments sampled at D1(+) MSNs from CHR2+ terminals in mice injected with AAV-ChR2 or AAV-CaMKII-Cre-GFP in the PFC in drug free ACSF or post *ex vivo* cocaine. [2way Repeated Measures ANOVA, Interaction: $F(20, 152) = 2.516$. ACSF (4,7), *Ex vivo* coc (3,6), Cre (7,11), Cre +coc (3,7)]. No difference was observed +/- cocaine in either viral condition [See Supp. Table 4 for full statistics]. **B.** Summary time course of PFC-D1(+) DSE experiments in drug free ACSF or following *ex vivo* cocaine. **C.** Maximum DSE evoked in either condition [gray bar in DSE summary graphs; ACSF(6,9) vs cocaine (3,6), $p<0.05$, student's *t*-test]. **D.** Summary time course of PFC-D1(+) DSE experiments from mice injected with Cre in drug free ACSF or following *ex vivo* cocaine. **E.** Maximum DSE evoked in either condition [Cre (4,7) vs. Cre +coc (3,7), $p>0.05$, student's *t*-test]. **F.** Optically evoked PPR curve experiments sampled at D1(-) MSNs from CHR2+ terminals in mice injected with AAV-ChR2 or AAV-CaMKII-Cre-GFP in the PFC in drug free ACSF or post *ex vivo* cocaine. [2way ANOVA, Interaction, $p<0.001$, $F(20, 152)=2.516$; ACSF (6,10), *Ex vivo* cocaine (4,6), Cre (7, 10), Cre +coc (3,7). Tukey's Posttest of ACSF vs. *Ex vivo* cocaine: 20ms $p<0.0001$, 50ms $p<0.0001$, 100ms $p<0.0001$, 200ms $p<0.05$]. **G.** Summary time course of PFC-D1(-) DSE experiments in drug free ACSF or following *ex vivo* cocaine. **H.** Maximum DSE evoked in either condition [ACSF(5,9) vs *Ex vivo* cocaine (4,6), $p<0.05$, student's *t*-test). **I.** Summary time course of PFC-D1(-) DSE experiments from mice injected with Cre in drug free ACSF or following *ex vivo* cocaine. **J.** Maximum DSE evoked in either condition (Cre (5,8) vs. Cre +coc (3,6), $p>0.05$, student's *t*-test).

These findings suggest that post-synaptic perturbation of NAc eCB production by cocaine exposure may restructure cortical output to the NAc in order to favor reward association. The reduction of release probability selectively at PFC-D1(-) synapses, as well as the reduction of DSE amplitude possibly via occlusion or inhibition of eCB production following acute cocaine demonstrates a shift in cortical control of NAc circuitry that would favor increased recruitment of D1(+) MSNs via a presynaptic eCB mechanism (**Supplementary Fig 4.7**). Enhanced D1(+) MSN activation has been repeatedly shown to favor reward acquisition (Lobo *et al*, 2010; Pascoli *et al*, 2014; Pascoli *et al*, 2015). Thus, as lacking CB1Rs in cortical projection neurons prevents these physiological effects of cocaine, the lack of CPP acquisition may be due to an inability to restructure cortical control over NAc output. Together, this suggests a potential mechanism by which exposure to salient stimuli restructures neurological circuits driving motivated behavior to influence future behavioral outcomes.

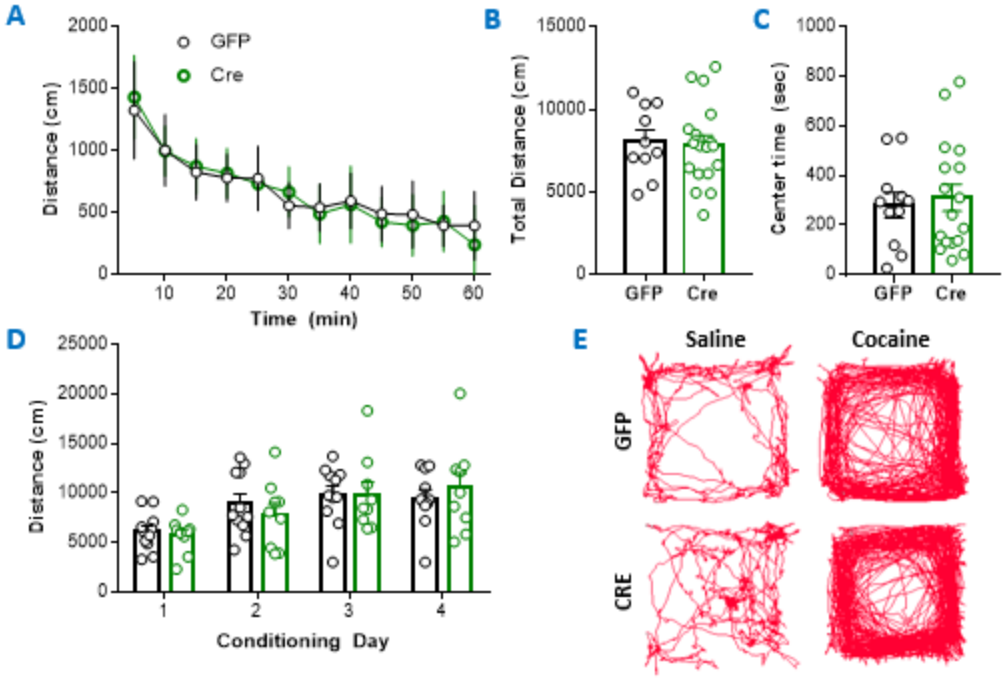
4.3 Supplementary Figures



Supplementary Figure 4.1 - Blocking CB1R component of DSE reduces but does not block the effect. **A.** Representative DSE experiment from a D1(+) MSN in ACSF. This and following experiments are normalized to average baseline EPSC preceding depolarization indicated by dashed vertical lines. **B.** Representative DSE experiment from a D1(+) MSN performed in the presence of the CB1R antagonist AM251 (5μM). **C.** Summary timecourse of DSE experiments performed in AM251 at D1(+) MSNs (red) compared to drug-free conditions (grey, data also shown in Fig. 2b). **D.** Summary of maximum DSE evoked in either condition [AM251 vs ACSF, $p<0.01$, *t*-test; AM251 81.45 ± 3.716 (5,8); ACSF 62.4 ± 4.82 (6,9)]. **E.** Representative DSE experiment from a D1(-) MSN in ACSF. **F.** Representative DSE experiment from a D1(-) MSN performed in AM251 (5μM). **G.** Summary time course of DSE experiments performed in AM251 at D1(-) MSNs (red) compared to drug-free conditions (grey, data also shown in Fig. 2g). **H.** Summary of maximum DSE evoked in either condition [AM251 vs ACSF, $p<0.01$, *t*-test; AM251 74.74 ± 4.974 (5,7). ACSF 54.29 ± 3.271 (5,9)].



Supplementary Figure 4.2 - Expression of CPP persists for four weeks following final test session. **A.** Change in time spent on cocaine-paired side in GFP injected (black) and CRE injected (green) mice following a four week incubation after conditioning test [2way Repeated Measures ANOVA, Sidak's multiple comparisons posttest, 2way ANOVA; GFP(10) vs. Cre (7), $p<0.05$. Full statistics in Supplementary Table 2]. **B.** Representative heat map images of time spent in each area of the test chamber during incubation test.



Supplementary Figure 4.3 - Removal of PFC CB1Rs does not impair basal locomotion or sensitization to cocaine. **A.** Binned data of distance traveled in a one hour open field (habituation) assay. **B.** Cumulative distance traveled in an open field assay. **C.** Time spent in the center of the arena during open field assay. **D.** Distance traveled in conditioning chamber following 15mg/kg IP cocaine injection. Animals in both groups displayed enhanced locomotor responding to cocaine following repeated exposure (2way Repeated Measures ANOVA. Time, $p<0.0001$ $F(3,54)=1.392$. Sidak's Multiple Comparisons Test of individual days compared to Day 1 cocaine: GFP- D2, $p<0.001$, D3, $p<0.0001$, D4, $p<0.0001$; Cre: D2, $p<0.05$, D3, $p<0.0001$, D4, $p<0.0001$. Complete statistics shown in Supplementary Table 1). **E.** Representative activity traces from GFP injected (Top row) and CRE injected (Bottom row) mice on the first saline exposure (Left) and following the final (fourth) pairing with cocaine (Right). Images represent the last 10 minutes of recorded activity.

Supplementary Table 4.1. 2way Repeated Measures ANOVA Results for cocaine-induced hyperlocomotion

	Sum of squares	DF	MS	F (DFn, DFd)	*p*
Interaction	11774196	3	3924732	F (3, 54) = 1.392	P=0.2551
Session	213923674	3	71307891	F (3, 54) = 25.29	P<0.0001
Virus	256660	1	256660	F (1, 18) = 0.008285	P=0.9285
Subjects	557647741	18	30980430	F (18, 54) = 10.99	P<0.0001

Sidak's multiple comparisons test, Day 1 vs. Day (x)					
		Mean Diff.	95.00% CI of diff.	Significant?	P value
GFP	Day 2	-2856	-4621 to -1092	Yes	P<0.001
	Day 3	-3718	-5482 to -1953	Yes	P<0.0001
	Day 4	-3391	-5155 to -1627	Yes	P<0.0001
Cre	Day 2	-2145	-4095 to -194.3	Yes	P<0.05
	Day 3	-4160	-6110 to -2210	Yes	P<0.0001
	Day 4	-4807	-6757 to -2856	Yes	P<0.0001
		Avg GFP (N)	Avg CRE (N)	T	Df
GFP	Day2 v. 1	6104 (11)	8690 (11)	3.99	54
	Day3 v. 1	6104 (11)	9821 (11)	5.193	54
	Day4 v. 1	6104 (11)	9495 (11)	4.736	54
Cre	Day2 v. 1	5703 (9)	7848 (9)	2.71	54
	Day3 v. 1	5703 (9)	9863 (9)	5.256	54
	Day4 v. 1	5703 (9)	10510 (9)	6.073	54

Supplementary Table 4.2. 2way Repeated Measures ANOVA Results for cocaine CPP experiments (Pre-conditioning KO)

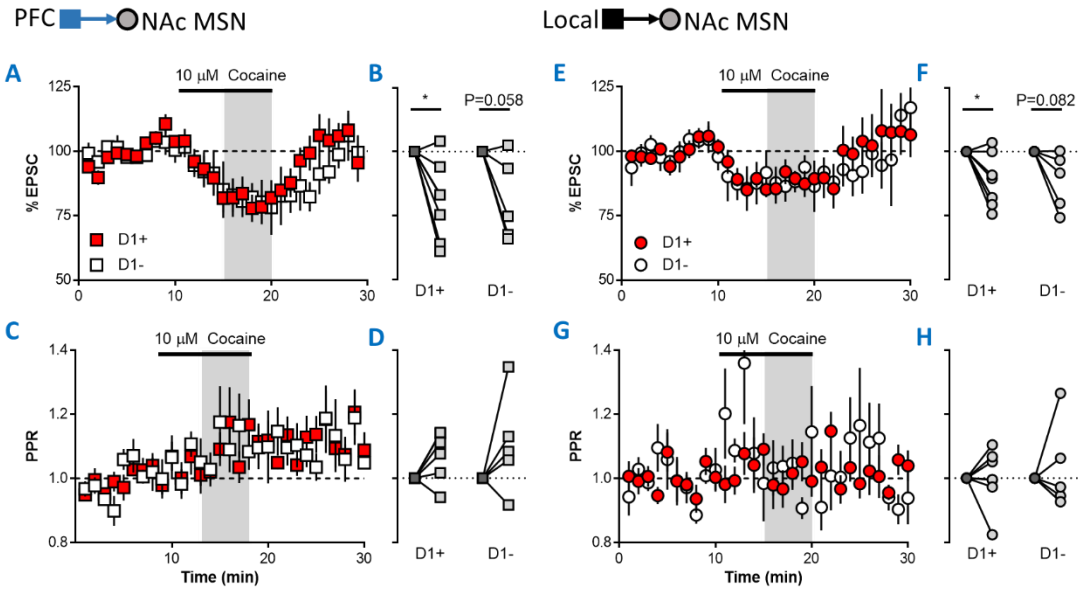
	Sum of squares	DF	MS	F (DFn, DFd)	*p*
Interaction	1.112	1	1.112	F (1, 15) = 6.209e-005	P=0.9938
Session	36324	1	36324	F (1, 15) = 2.029	P=0.1748
Virus	460815	1	460815	F (1, 15) = 9.518	P=0.0075
Subjects	726251	15	48417	F (15, 15) = 2.704	P=0.0316

Sidak's multiple comparisons test, GFP(10) vs. Cre (7)				
Session	Mean Diff.	95.00% CI of diff.	Significant?	P value
Conditioning	236.2	24.94 to 447.4	Yes	0.0264
4wk Incubation	236.9	25.67 to 448.2	Yes	0.0259
Details	Avg GFP (N)	Avg CRE (N)	T	Df
Conditioning	315.8 (10)	79.61 (7)	2.632	30
4wk Incubation	249.7 (10)	12.83 (7)	2.64	30

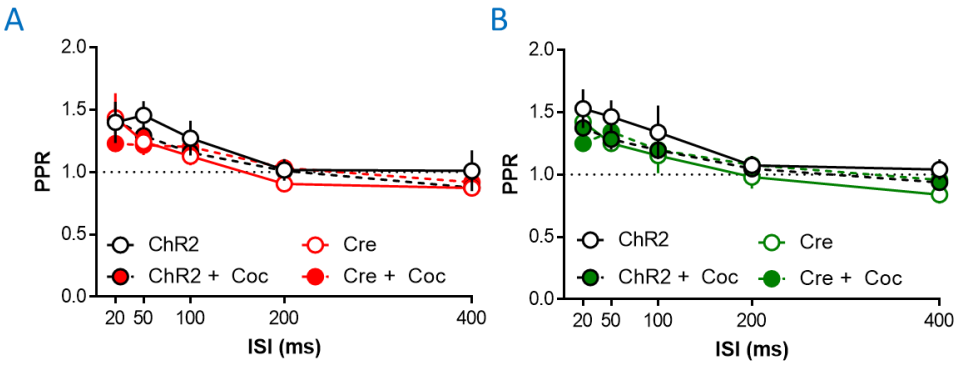
Supplementary Table 4.3. 2way Repeated Measures ANOVA Results for cocaine CPP experiments (Post-conditioning KO)

	Sum of squares	DF	MS	F (DFn, DFd)	*p*
Interaction	84236	1	84236	F (1, 11) = 2.251	P=0.1617
Session	111694	1	111694	F (1, 11) = 2.984	P=0.1120
Virus	2753	1	2753	F (1, 11) = 0.04224	P=0.8409
Subjects	716919	11	65174	F (11, 11) = 1.741	P=0.1858

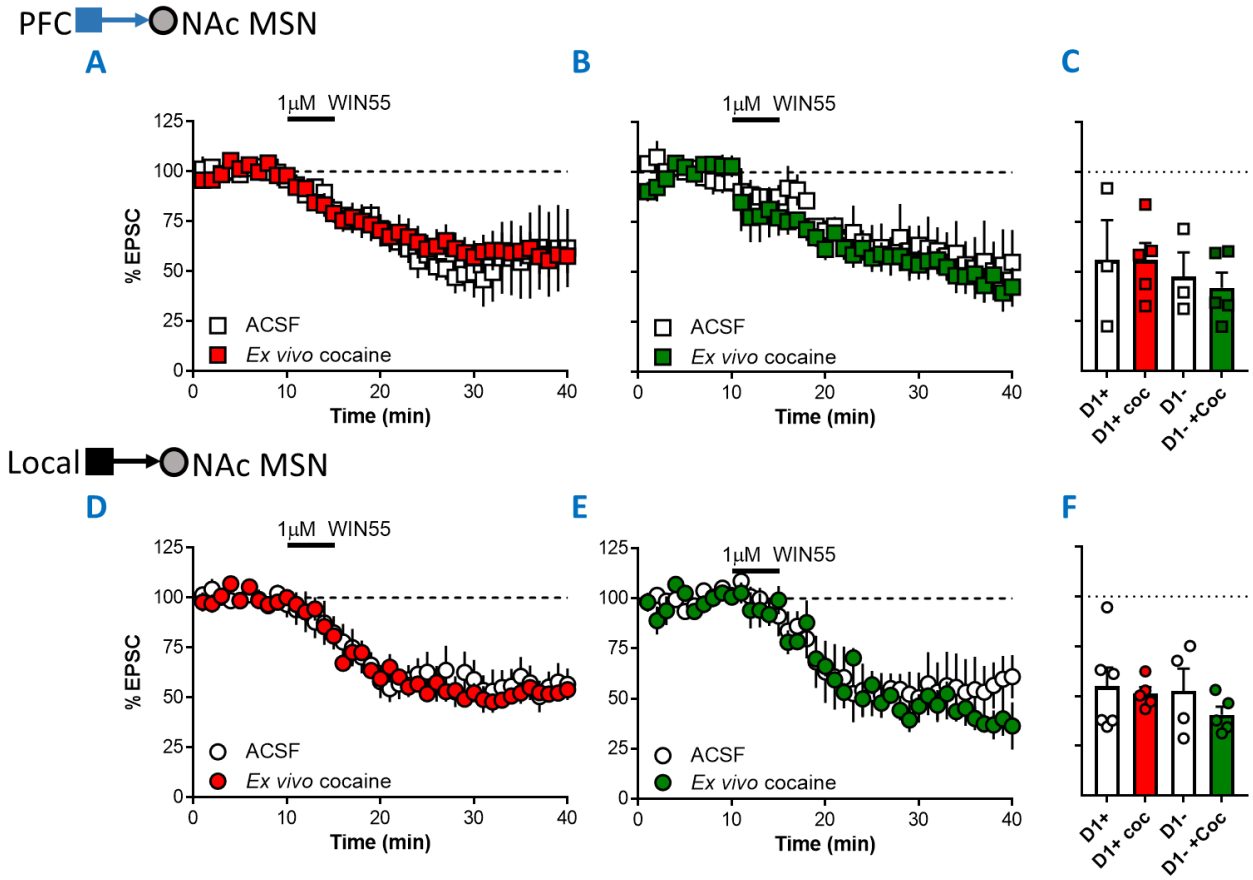
Sidak's multiple comparisons test, GFP(6) vs. Cre (7)				
Session	Mean Diff.	95.00% CI of diff.	Significant?	P value
Conditioning	134.8	-167.6 to 437.2	No	0.5048
4wk Incubation	-93.54	-395.9 to 208.8	No	0.7146
Details	Avg GFP (N)	Avg CRE (N)	T	Df
Conditioning	481.7 (6)	346.9 (7)	1.07	22
4wk Incubation	236.1 (6)	329.6 (7)	0.7423	22



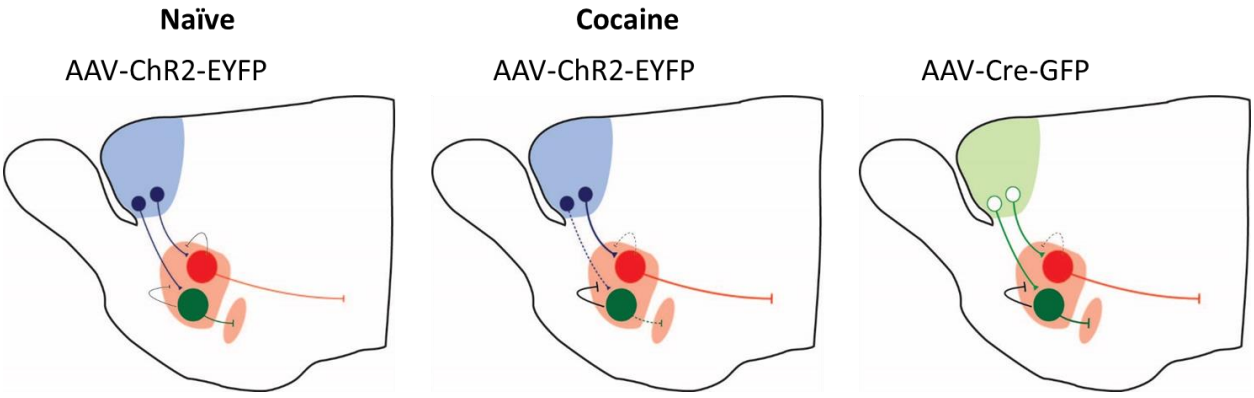
Supplementary Figure 4.4 - *Ex vivo* cocaine transiently decreases EPSC amplitude. **A.** Bath application of cocaine (10µM) induces a transient reduction in optically-evoked EPSCs at D1(+) MSNs and a trend towards a reduction in amplitude at D1(-) MSNs. **B.** %Change in EPSC amplitude. Values indicate baseline (100%) and average of the last five minutes of cocaine (grey bar, previous graph) [One sample *t* test. D1+ 80.73 ± 5.841 (5, 7), P<0.05. D1- 80.84 ± 7.298 (4, 5), P=0.058]. **C.** Cocaine-induced decrease in optically-evoked EPSCs does not change paired pulse ratio. **D.** Normalized change in PPR. Values indicate baseline (1.0) and average of the last five minutes of cocaine (grey bar, previous graph) [One sample *t* test. D1+ 1.123 ± 0.058 (5, 7), P>0.05. D1- 1.107 ± 0.069 (4, 5), P>0.05]. **E.** Bath application of cocaine (10µM) induces a small transient reduction in electrically-evoked EPSCs at D1(+)and a trend towards a reduction in amplitude at D1(-) MSNs. **F.** %Change in EPSC amplitude. Values indicate baseline (100%) and average of the last five minutes of cocaine (grey bar, previous graph) [One sample *t* test. D1+ 88.7 ± 3.964 (5, 7), P<0.05. D1- 88.51 ± 4.974 (4, 5), P=0.082]. **G.** A change in PPR does not accompany cocaine-induced decrease in electrically-evoked EPSC amplitude. **H.** Normalized change in PPR. Values indicate baseline (1.0) and average of the last five minutes of cocaine (grey bar, previous graph) [One sample *t* test. D1+ 1.001 ± 0.034 (5, 7), P>0.05. D1- 1.034 ± 0.062 (4, 5), P>0.05].



Supplementary Figure 4.5 - Neither removal of CB1Rs from cortical afferents nor bath application of cocaine affects PPR evoked using electrical stimulation. **A.** Summary PPR experiments evoked using electrical stimulation at D1(+) MSNs. [2way Repeated Measures ANOVA. Interaction: F (20, 152) = 0.9785, P=0.4911. Condition: F (5, 38) = 0.1937, P=0.9631. Mice/cells: ChR2 (9,13), Cre (5,7), ChR2 + Coc (3,6), Cre + Coc (3,7)]. **B.** Summary PPR experiments evoked using electrical stimulation at D1(-) MSNs [2way Repeated Measures ANOVA. Interaction: F (20, 184) = 0.8226, P=0.6842. Condition: F (5, 46) = 0.4496, P=0.8114. Mice/cells: ChR2 (9,10), Cre (5,8), ChR2 + Coc (4,6), Cre + Coc (3,10)].



Supplementary Figure 4.6 - Reduction in EPSC amplitude mediated by the CB1R agonist WIN55 (1μM) is not affected by *ex vivo* cocaine. **A, B.** WIN55 mediated reduction of optically-evoked EPSC amplitude at D1+ or D1- MSNs in ACSF (white squares) or following *ex vivo* cocaine (red, green squares). **C.** Summary of WIN55-mediated reduction of optical EPSC amplitude [One sample *t* test vs. 100. D1+: ACSF, 55.66 ± 20.06 (3,3), P=0.157. *Ex vivo* cocaine, 55.71 ± 8.609 (4,5), P<0.01. D1-: ACSF 47.41 ± 12.22 (3,3), P<0.05. *Ex vivo* cocaine, 41.8 ± 7.6 (5,5), P<0.01]. **D, E.** WIN55 mediated reduction of electrically-evoked EPSC amplitude at D1+ or D1- MSNs in ACSF (white circles) or following *ex vivo* cocaine (red, green circles). **F.** Summary of WIN55-mediated reduction of electrical EPSC amplitude [One sample *t* test vs. 100. D1+: ACSF, 54.87 ± 9.447 (6,6), P<0.01. *Ex vivo* cocaine, 51.23 ± 3.186 (4,5), P<0.01. D1-: ACSF 52.62 ± 11.15 (4,4), P<0.05. *Ex vivo* cocaine, 40.59 ± 4.029 (5,5), P<0.001].



Supplementary Figure 4.7 - Model of eCB recruitment at PFC terminals in the NAc by cocaine. In naïve animals, eCBs exert control over cortical inputs onto both D1(+) and D1(-) MSNs. Following *ex vivo* cocaine, eCBs are recruited to tonically inhibit release probability at PFC-D1(-) synapses, likely occluding DSE. At PFC-D1(+) synapses, release probability is unchanged but DSE is reduced demonstrating a reduction in eCB signaling. This likely biases throughput of PFC-NAc transmission towards a pro-reward (D1(+) driven) state. In mice lacking CB1Rs at these terminals, *ex vivo* cocaine is unable to induce this shift in the circuit which correlates with a lack of CPP generation.

4.4 Methods

Animals – All animals were bred and housed at Vanderbilt under the supervision of the Department of Animal Care. Conditional *cnr1fl/fl* mice (Dr. Eric Delpire) were crossed to B6;129S-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J and BAC Drd1a-tdTomato mice to obtain the triple transgenic cnr1fl/fl-chr2fl/fl-tdTom mouse line. Animals were back-crossed for six generations prior to use in experiments. Animals were housed on a 12-hour light/dark cycle and fed ad lib. Breeding cages were given access to 5LOD chow (PicoLab®, 28.7% Protein, 13.4% Fat, 57.9% Carbohydrate) to improve the viability of litters. Upon weaning at P21-28, experimental animals were switched to standard chow.

Stereotaxic surgery - All surgeries were performed in accordance to guidelines set by Vanderbilt IACUC. Briefly, 4-6 week male C57BL6 mice (pre-conditioning) or mice post conditioning (7 weeks) were anesthetized using a cocktail of ketamine (75 mg/kg) and dexdomitidor (0.5 mg/kg). Craniotomies were performed using a manual drill, AmScope microscope, and World Precision Instruments Aladdin Al-2000 syringe pump hydraulic system. Injection sites were based on coordinates listed in The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008). The PFC (AP 1.4, ML ± 0.5, DV -2.9 mm) was located using the Leica AngleTwo Stereotaxic software. AAV-CamKII-ChR2-EYFP, AAV-CamKII-Cre-GFP, or AAV-CamKII-GFP (UNC Vector Core) was injected at 100 nL per minute and allowed to permeate into the tissue for 10 minutes before removal of the syringe. Mice were revived using 0.5 mg/kg antisedan and treated with 5 mg/kg ketoprofen up to three days following surgery as needed.

Behavior - Behavior was performed in MedAssociates Activity Test Chambers. Mice used in cocaine conditioned place preference (CPP) experiments were habituated to the behavior chambers in a one hour open field test. On the following day, chambers were modified to create two sides

based on visual and texture cues. Mice were placed into the chamber and allowed to explore freely to establish an initial preference (pretest) for 20 minutes. On subsequent conditioning sessions, mice were given a single injection of vehicle (saline) or cocaine (15 mg/kg) IP immediately prior to being placed in the chamber with both sides having the same contextual cues. Two conditioning sessions were performed each day (AM and PM), alternating cocaine and saline order each day with at least a four hour window between sessions. Side pairings were determined using a biased design with the less preferred side being paired with cocaine. Following posttest, mice were housed in home cages for four weeks prior to assessing preference maintenance. For post-conditioning knockout experiments, cohorts were subjected to four conditioning sessions and stereotaxic surgery performed within 24 hours after the test session. Locomotor activity was tracked using Noldus Ethovision software.

Electrophysiology – Mice were anesthetized using isoflurane prior to sacrifice and parasagittal sections (250 μm) containing the NAc were prepared from whole brain tissue using a Leica Vibratome as previously described (Self cite). Briefly, slices were placed in an N-methyl D-glucamine (NMDG) based recovery solution (2.5 KCL, 20 HEPES, 1.2 NaH2PO4, 25 Glucose, 93 NMDG, 30 NaHCO¬3, 5.0 sodium ascorbate, 3.0 sodium pyruvate, 10 MgCl2, and 0.5 mM CaCl2-2H2O) for 12-15 min at 32°C before transfer to a chamber containing artificial cerebral spinal fluid (ACSF, 119 NaCl, 2.5 KCL, 1.3 MgCl2-6H2O, 2.5 CaCl2-2HO, 1.0 NaH¬2¬PO¬4¬-H¬2¬O, 26.2 NaHCO¬3, and 11 mM glucose) until use. Patching performed using a Scientifica Slicescope Pro System under a constant perfusion of 32°C ACSF at a rate of 2 mL/min. MSNs were visualized with a Scientifica PatchVision software. Patch pipettes were 3-5 MΩ were pulled using a P1000 Micropipette Puller and filled with a potassium gluconate based internal solution [125 mM, 4 NaCl, 10 HEPES, 4 Mg2+ATP, 0.3 Na+GTP, and 10 mM Na+ phosphocreatine] for

DSE, PPR, and agonist experiments. Pipettes were filled with a Cs+ internal solution (120 CsMeSO3, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4.0 Mg2-ATP, 0.3 Na2-GTP, 0.1 spermine, and 5.0 mM QX 314 bromide) for AMPA/NMDA experiments. MSNs were identified based on visual appearance (size, morphology) as well as resting membrane potential; MSNs were identified as D1+ or D1- by fluorescence of tdTomato. All experiments were performed in the presence of the GABAA channel blocker picrotoxin (50 μM). Stimulation control of light and electrical stimulation as well as data collection was performed using Molecular Devices pClamp 10 Analysis software. Cell electrical properties were monitored using an Axopatch 500B Multiclamp amplifier and Axon Digidata 1550 low-noise data acquisition digitizer. Responses were filtered at 2 kHz and digitized at 10 kHz. Optical stimulation of ChR2-expressing terminals was performed using a CoolLED pE-100 LED excitation system. 480 nm light was pulsed through the high-powered (40x) objective to excite ChR2+ terminals at 0.1 – 0.2 Hz for 0.5-1 msec. Light intensity was adjusted to evoke stable responses. Paired stimuli were delivered at 20, 50, 100, 200, and 400 ms to generate the paired pulse ratio curves. Depolarization induced suppression of excitation (DSE) was evoked by depolarizing patched MSNs to +30mV for 10 seconds. For cocaine wash-on experiments, Cocaine-HCl was dissolved in H2O at 20mM and diluted to 10μM in ACSF. Stock solutions were prepared fresh weekly. Cocaine was washed on for 10 minutes while recording. PPR and DSE experiments were performed at least 10 minutes following removal of drug from the bath solution. NMDA currents in AMPA/NMDA ratios were determined by the peak amplitude 50ms post onset of the +40mV EPSC.

Drugs - Ketoprofen, dexdomitodor, ketamine, and antisedan were obtained from Patterson Veterinary Supplies. Picrotoxin and cocaine hydrochloride were obtained from Sigma Aldrich. WIN55 was obtained from Tocris Bioscience. WIN55 and picrotoxin were dissolved in DMSO.

Stocks were sufficiently concentrated to keep the concentration of DMSO in ACSF <0.04%.

Data Analysis – Electrophysiology experiments were analyzed using using Clampfit 10.4, Microsoft Excel, and Graphpad Prism v6.0. For agonist experiments, change in baseline and PPR were calculated by averaging values in the highlighted regions and comparing to the average across baseline events. Values were normalized prior to analyses. One-sample t-tests were performed to determine significant changes in EPSC amplitude and PPR in the given window. Two-tailed t-tests were used to compare DSE amplitude in the given contexts. PPR was analyzed using a 2way ANOVA across all groups with Dunnett's post test to determine differences from controls (ChR2 – only). CPP was analyzed by subtracting time spent on the drug-paired side in pretests from posttest values (ΔCPP). Preference association was determined by comparing raw time on drug paired side to pretest values (students t-test). ΔCPP was analyzed using two-way repeated measures ANOVA with Sidak's posttest to compare individual days. Distance traveled in cocaine (15 mg/kg) conditioning sessions was analyzed with a 2-way ANOVA and Dunnett's posttest. For all analyses, alpha was set as 0.05. Number of experiments is listed as number of animals per group and total number of cells respectively (N, n).

CHAPTER 5

Striatal knockout of cannabinoid type-1 receptors in A2a neurons disrupts retention of cocaine-environment association

5.1 Introduction

Substance use disorders are widespread, costly, and resistant to treatment due to the high propensity of relapse (Joffe *et al*, 2014; Turner *et al*, 2018; Volkow *et al*, 2015). Progression from drug use to abuse is tied to maladaptive changes in forebrain synaptic transmission particularly within the mesolimbic dopamine system. These forebrain circuits are heavily regulated by a host of modulatory cascades including the endocannabinoid system (eCB). Endogenous cannabinoid signaling within forebrain regions is extensively implicated in the coordination of adaptive and maladaptive reward seeking towards drugs of abuse (Fourgeaud *et al*, 2004; Gremel *et al*, 2016; Grueter *et al*, 2010; McCutcheon *et al*, 2011a; Robbe *et al*, 2002; Thiemann *et al*, 2008; Wolf, 2016). Targeting of projection-specific forebrain eCB signaling may be a useful intervention for substance use disorders.

The canonical target of eCBs are presynaptic CB1Rs. Pharmacological inhibition of CB1Rs opposes reward seeking and CB1R knockout-mice are resistant to the reinforcing effects of psychostimulants (Soria *et al*, 2005; Yu *et al*, 2011). Targeted removal of CB1Rs using conditional knockout mouse lines has suggested the impact of eCB signaling on drug-reward behaviors varies based on its action at excitatory and inhibitory synaptic connections. Notably, forebrain CB1Rs expressed in glutamatergic and GABAergic neurons exert bidirectional control over hedonic behavior. Removal of CB1Rs in glutamatergic projection neurons attenuates the

potentiation of hedonic feeding induced by the CB1R agonist THC while removing CB1Rs from GABA-ergic neurons opposes hedonic hypophagia induced by high doses of THC (Bellocchio *et al*, 2010). A similar dichotomy is seen with psychostimulant-induced behaviors; deletion of CB1Rs from GABAergic neurons inhibits responsiveness to cocaine while removing CB1Rs from glutamatergic neurons inhibits association of drug reward with external stimuli (Martin-Garcia *et al*, 2016). These findings highlight the need for additional studies examining how cell-type specific expression of forebrain CB1Rs affect psychostimulant reward.

Here, we utilized *Drd1a* (D1) and *Adora2* (A2a)-CRE recombinase mouse lines crossed with conditional $cnr1^{fl/fl}$ mice to generate cell-type specific knockouts of CB1Rs and examine how loss of CB1R signaling in specific GABAergic populations of forebrain neurons impacts cocaine-induced behaviors. We find that mice lacking CB1Rs in either D1 or A2a neurons exhibit a reduction in cocaine-induced hyperlocomotion and a lack of cocaine sensitization. Knockout of CB1Rs in both populations of neurons also impaired the retention but not development of cocaine conditioned place preference (CPP). These results indicate that CB1R signaling in GABAergic striatal neurons are necessary for cocaine-induced hyperlocomotion and the retention of cocaine-environment associations.

5.2 Results

Removal of CB1Rs from D1 and A2a neurons does not impair locomotor function

CB1R expression and/or function, particularly in the forebrain, is necessary for the acquisition and expression of psychostimulant associated behaviors (Martin-Garcia *et al*, 2016; Orio *et al*, 2009). Particularly, results have shown that CB1Rs in GABAergic neurons regulates sensitivity to cocaine. Therefore, we decided to expand upon the role of CB1R function in GABAergic neurons with respect to cocaine-associated behaviors adding cell-type specificity. We

chose to utilize *drd1*-cre and *Adora*-cre mice, which express cre-recombinase in D1-receptor neurons and Adenosine 2a receptor (A2a) positive neurons respectively, crossed to a conditional *cnr1* mouse line to selectively knockout CB1Rs in D1 and A2a neuronal populations (D1-*cnr1* and A2a-*cnr1*, **Fig. 5.1a**). Both D1 and A2a receptors are enriched throughout the dorsal and ventral striatal circuitry (Kravitz and Kreitzer, 2012a; Kravitz *et al*, 2012b; Kreitzer *et al*, 2008) and have been extensively utilized to target 'direct' and 'indirect,' or midbrain and pallidal projecting medium spiny neurons (MSNs) (Joffe *et al*, 2017; Lobo *et al*, 2011; Tejeda *et al*, 2017; Wall *et al*, 2013). We initially examined whether removal of CB1R in either D1 or A2a neuronal populations affected locomotor activity using a one hour open field assay. Mice lacking CB1Rs in either cell-type did not display any locomotor deficits compared to littermate controls [One-way ANOVA, Distance: $F(2,34) = 0.334$, $p>0.05$; Center Time: $F(2,34) = 0.1363$, $p>0.05$, Entries: $F(2,34) = 0.413$, $p>0.05$, **Table 5.1**]. Knockout groups displayed nearly identical locomotor activity throughout the task (**Fig 5.1b,c**). Additionally, lacking CB1Rs did not affect time spent nor number of entries into the center of the chamber (**Fig 5.1d-f**) which is a proxy for anxiety. These results demonstrate that cell-type specific knockout of CB1Rs does not affect locomotor function.

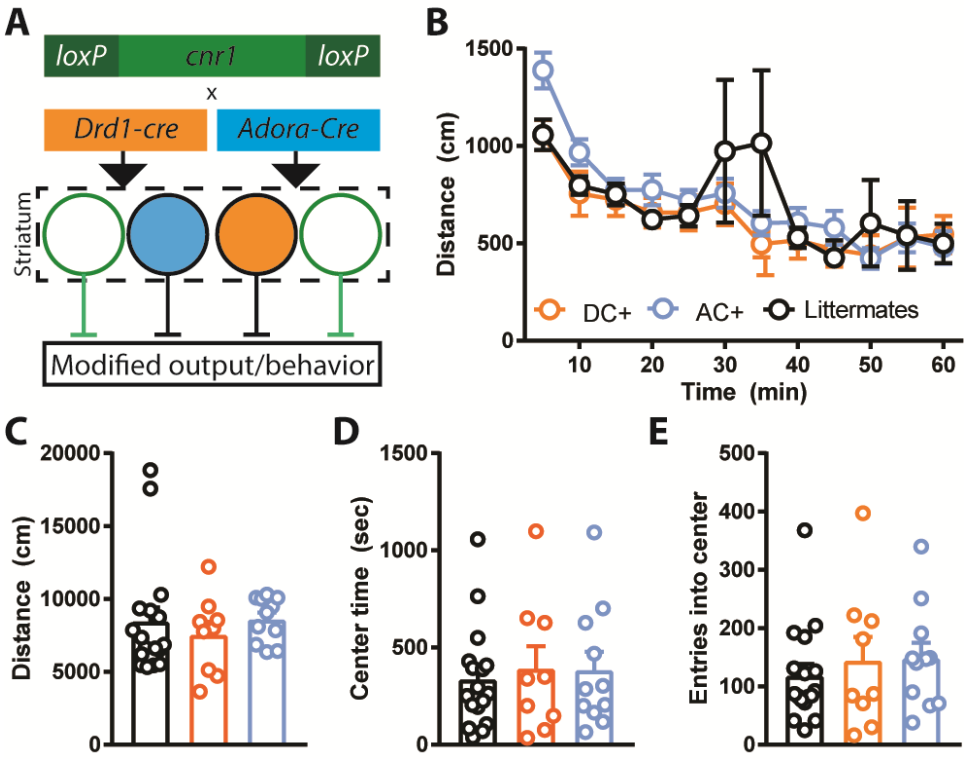


Figure 5.1 - Pathway-specific deletion of striatal CB1Rs does not alter locomotor activity.
A. Schematic of genetic approach to generate cell-type specific deletions of CB1Rs. **B.** Distance traveled over the course of open field assay in 5min bins. **C.** Cumulative distance traveled in 60 min open field assay. Distance did not vary with genotype. **D.** Cumulative center time did not differ by genotype. **E.** Number of entries into the center of the arena was not different across genotypes.

Table 5.1 - Descriptive statistics of Open Field Assay

Group (*n*)	***cnr1* (17)**	**DC-*cnr1* (9)**	**A2a-*cnr1* (11)**
Distance (cm)	8456 ± 962.2	7542 ± 888.3	8600 ± 465.8
Center Time (sec)	336.4 ± 63.84	391.4 ± 114.9	384.4 ± 93.99
Entries (#events)	118.4 ± 20.0	144.4 ± 40.4	148.4 ± 26.5

One-way ANOVA. Distance: $F(2,34) = 0.334$, $p>0.05$. Center Time: $F(2,34) = 0.1363$, $p>0.05$. Entries: $F(2,34) = 0.413$, $p>0.05$.
Values shown as mean ± SEM. Knockout vs. littermate controls were not significantly different in across any measure (One-way ANOVA, Dunnett's posttest).

CB1Rs in both D1 and A2a neurons are necessary for cocaine locomotor sensitization

We next examined whether D1-*cnr1* or A2a-*cnr1* mice exhibited differences in the hyperlocomotive response to cocaine using a cocaine-sensitization assay (**Fig 5.2a**). Mice were habituated to the injection (Vehicle, saline) and activity chambers over two days. Following habituation, mice were given a 15mg/kg injection of cocaine immediately prior to placement in the chamber for five days while locomotor activity was assessed. Littermate controls exhibited a robust increase in locomotor activity over the five day sensitization period (**Fig 5.2b,c**, T**able 5.2**). However, both D1- and A2a-*cnr1* mice did not exhibit significant sensitization when compared to the first day of cocaine (**Table 5.2**). Both knockout groups also exhibited decreased locomotor activity when compared to littermate controls. These findings demonstrate that CB1Rs on both D1 and A2a MSNs are necessary for the hyperlocomotive effects of cocaine.

Table 5.2 - Distance traveled per session in sensitization task [Distance (cm) ± SEM]

Group (*n*)	***cnr1* (13)**	**DC-*cnr1* (8)**	**A2a-*cnr1* (9)**
Saline - 1	****1702 ± 190.6	**1219 ± 172.9	1373 ± 132.5
Saline - 2	****1608 ± 176.4	**1303 ± 161.1	1241 ± 105.4
Cocaine - 1	6769 ± 742.4	2654 ±230.5	5114 ± 831.3
Cocaine - 2	*9533 ± 1264	4594 ± 458.5	5970 ± 820.6
Cocaine - 3	**10169 ± 1488	4646 ± 634.6	6673 ± 1133
Cocaine - 4	***10880 ± 1581	5135 ± 805.8	6808 ± 798.3
Cocaine - 5	***10948 ± 1822	4536 ± 474.9	7260 ± 838.7
Cocaine - re-exposure	****12143 ± 1958	5715 ± 1023	7636 ± 1536

Significant interaction of day and genotype (2way ANOVA, $F(14, 189) = 3.018$, $p<0.001$). Asterisks denote significance vs. Cocaine – 1 (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, Dunnett's posttest).

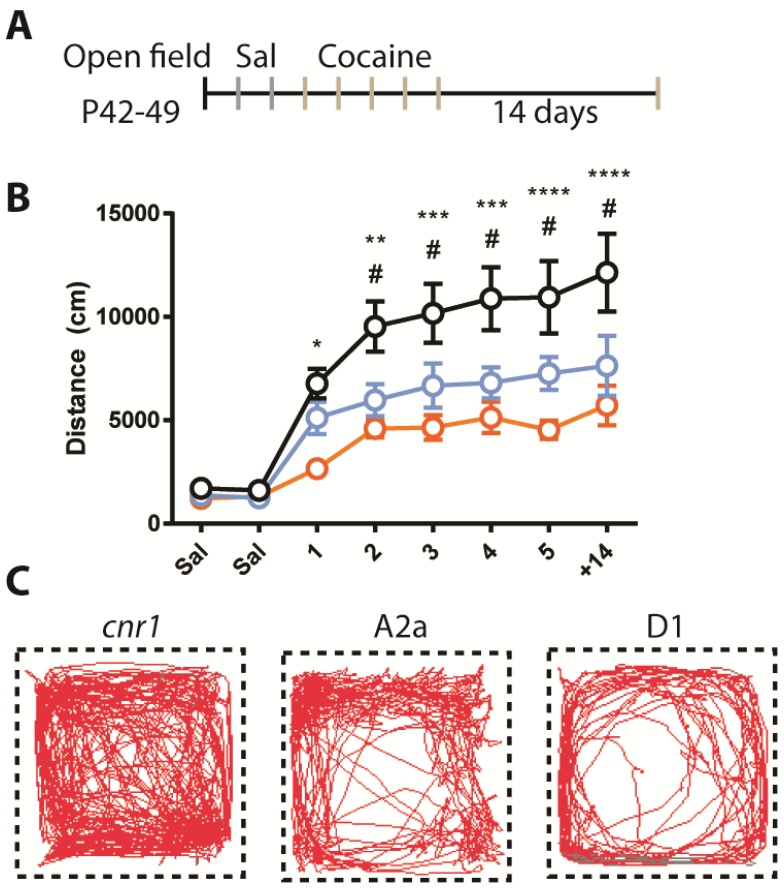


Figure 5.2 - Deletion of striatal CB1Rs in either D1 or A2a+ MSNs reduces cocaine locomotor activity and impairs sensitization. **A.** Schematic of cocaine sensitization paradigm. **B.** Distance traveled per session in cocaine (15mg/kg) sensitization assay. DC-*cnr1* (orange circles) and A2a-*cnr1* (blue circles) mice were significantly less active than their WT littermates (black circles) following cocaine exposure (2way ANOVA of distance traveled/day: Interaction $F(14, 189) = 3.018$, $p<0.001$. A2a and DC mice did not sensitize to cocaine. A2a mice were significantly different from littermate controls by day 2 [#, $p<0.05$] and DC-*cnr1* by on day 1 [*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$, Dunnett's posttest). **C.** Representative activity diagrams of the final cocaine exposure session (+14). Traces represent the last 10 minutes of the session.

CB1R expression in D1 and A2a neurons is necessary for the maintenance of cocaine conditioned place preference

CB1R expression in glutamatergic and GABAergic neurons has been shown to regulate distinct aspects of psychostimulant-induced behaviors (Martin-Garcia *et al*, 2016). Having established that CB1Rs in *D1* and *A2a* neurons are necessary for cocaine locomotor sensitization, we next sought to determine whether CB1R expression in either cell-type was also necessary for drug-environment associative memory formation using a conditioned place preference (CPP) assay. We chose to assay preference formation following a single exposure, repeated exposure, and a putative 'abstinence' time point to observe any effects in the development or retention of a preference. Following a single pairing of saline/cocaine with the non-preferred side of the chamber, all animals failed to develop a significant preference for the drug paired side (**Fig 5.3a, Table 5.3**). Following repeated exposure, littermate controls and both knockout groups exhibited a significant preference for the drug paired side (**Fig 5.3b, Table 5.3**). This coincided with similar performances in a sucrose preference assay in which animals from all groups displayed a robust preference for sucrose over unsweetened water (**Fig 5.3D**). Together, these results suggest that CB1Rs in D1 and A2a neurons are not necessary for the preference formation to either drug or non-drug rewards. However, following a four week abstinence period from the cocaine CPP chambers, littermate controls retained a robust preference for the drug paired side (**Fig 5.3c, Table 5.3**), while *A2a-cnr1* and *D1-cnr1* animals did not. These results suggest that CB1R expression in both cell-types is required for the persistence of drug-environment association but not the formation of the initial preference.

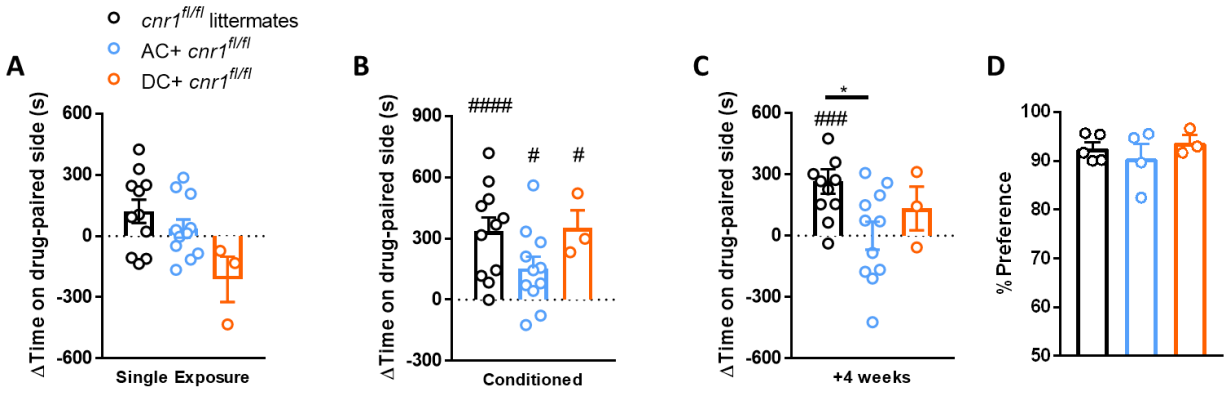


Figure 5.3 - Lacking CB1Rs in D1 or A2a neurons impairs retention of cocaine conditioned place preference. The same animals are represented in panels A-C across different time points in the CPP paradigm. **A.** Change in time spent on the drug paired side after a single pairing of cocaine. **B.** Change in time spent on the drug paired side after three additional pairings with cocaine. All groups developed a significant preference to the drug paired side [2way repeated measures ANOVA of time on drug paired side (sec): Interaction F (6, 66) = 3.41, $p<0.01$; Dunnett's post-test, #, $p<0.05$**, ####,** $p<0.0001$. Data points in panels A-C are continuous and represent multiple test sessions in the same animals]. **C.** Maintenance of CPP after a four week absence from the test chamber (2-way ANOVA of ΔCPP Times: Interaction: F (6, 66) = 2.347, $p<0.05$. WT vs AC-CNR1 (4 weeks), * $p<0.05$, Bonferroni's posttest. ###, $p<0.001$, Dunnett's post-test). **D.** %Preference for 2% sucrose solution over 24 hours [*cnr1*, 92.57 ± 1.23(5), AC x *cnr1*, 90.56 ± 3 (4), DC x *cnr1*, 93.75 ± 1.491 (3). No significant difference between groups].

Table 5.3 - Total time spent on drug paired side in CPP assay [Time (sec) ± SEM]

Group (*n*)	***cnr1* (11)**	**DC-*cnr1* (3)**	**A2a-*cnr1* (11)**
Pretest	448.72 ± 27.06	494.65 ± 50.47	482.73 ± 27.35
Single Exposure	570.39 ± 57.51	282.19 ± 73.15	519.16 ± 56.67
Repeat Conditioning	####783.57 ± 61.76	#845.76 ± 67.53	#635.77 ± 66.30
4week Maintenance	###714.37 ± 50.19	627.65 ± 101.6	482.46 ± 69.77

Significant interaction of day and genotype (2way ANOVA, F (6, 66) = 3.41, $p<0.01$). Asterisks denote significance vs. *Pretest* (#: $p<0.05$, ###: $p<0.001$, ####: $p<0.0001$, Dunnett's posttest).

5.3 Discussion

The dorsal striatum boasts the highest concentration of CB1Rs in the brain. In this region, CB1Rs are widely expressed on MSNs and various interneuron populations (Mathur *et al*, 2013; Van Waes *et al*, 2012). CB1R expression and function within striatal forebrain circuits are closely tied to locomotor activity as well as goal directed behavior and habits (Gremel *et al*, 2016; Monory *et al*, 2007). Additionally, numerous studies have examined the importance of CB1Rs with respect psychostimulant use/abuse (Gerdeman *et al*, 2002; Gremel *et al*, 2016; Gremel *et al*, 2013; Mathur and Lovinger, 2012). However, the role of CB1Rs on distinct populations of forebrain striatal neurons is unclear. We utilized two cre-recombinase mouse lines, *D1*- and *A2a*-cre, to target distinct GABAergic neuronal populations within the striatum (Joffe *et al*, 2017; Lobo *et al*, 2011) and knockout CB1Rs in a cell-type specific manner. It should be noted that *D1*-cre can target other neurons including *D1*-expressing principal neurons in the cortex and amygdala. However, the high density of CB1R expression within the striatum and the largely non-overlapping phenotypes exhibited by *D1-cnr1* and *Glu-cnr1* knockouts (Monory *et al*, 2007) suggest that any phenotype arising in D1-*cnr1* mice is likely due effects in striatal D1 MSNs. Additionally, A2a-expression is primarily confined to the striatum (Weaver DR 1993) suggesting observations within this study are likely due to modification of striatal circuits.

Surprisingly, removal of CB1Rs from either D1 or A2a neurons did not impact locomotor behavior (**Fig 1a,b**). Additionally, KO groups displayed no differences in center time indicating similar basal levels of stress compared to littermate controls (**Fig. 1c-e**). Other groups have demonstrated striatal cannabinoid signaling as important movement (Catlow and Sanchez-Ramos, 2015; Sanudo-Pena *et al*, 1999) as well as the generation of movement disorders such as Huntington's and Parkinson's (Blazquez *et al*, 2011; Kreitzer and Malenka, 2007; Mievis *et al*,

2011). However, these findings are consistent with an earlier report using glutamate, GABA, CaMKII, and D1 specific CB1R knockout lines which similarly showed no difference in locomotor activity at baseline (Bellocchio *et al*, 2010; Monory *et al*, 2007). Thus, while striatal CB1Rs are broadly involved in basal locomotion this involvement does not extend to CB1Rs on striatal MSNs.

We next sought to investigate whether D1- or A2a-*cnr1* mice responded differently to non-contingent cocaine administration. Interestingly, neither D1- nor A2a-*cnr1* groups sensitized to cocaine and displayed significantly less cocaine induced hyperolocomotion compared to littermate controls (**Fig 2b**). Unlike our findings, others have shown that CB1R-deficient mice display less activity in the presence of cocaine but still sensitize (Corbille *et al*, 2007). The lack of sensitization in our approach compared to the effect in global CB1R knockouts suggests that a balance of CB1R signaling across multiple cell types may be required for sensitization. In agreement with our findings, it has been previously demonstrated that co-administration of the CB1R antagonist AM251 also reduces cocaine-induced hyperlocomotion (Tozzi *et al*, 2012). Other groups have shown that similar inhibition of cocaine sensitization can be accomplished by antagonizing CB1Rs specifically within the nucleus accumbens (Caille *et al*, 2007; Mereu *et al*, 2015; Ramiro-Fuentes and Fernandez-Espejo, 2011). However nucleus accumbens MSNs do not primarily express CB1Rs (Winters *et al*, 2012) and is unlikely to be driving the lack of sensitization seen in the *D1-* and *A2a-cnr1* animals. Thus, these findings suggest a role for dorsal-striatal regulation by endocannabinoids that is independent of output target or requires simultaneous regulation of both pathways.

CB1Rs have been shown to control the formation and/or retention of cue-induced drug seeking. Global CB1R knockouts exhibit a lowers breakpoint for cocaine (Soria *et al*, 2005), an effect that can be mimicked by infusion of the CB1R antagonist SR141716A into the nucleus

accumbens. More relevant to this study, global pretreatment with a CB1R antagonist also inhibits the development but not expression of psychostimulant CPP (Yu *et al,* 2011). Interestingly, we found that *A2a-* and *D1-cnr1* mice were able to form a preference for cocaine in a CPP assay. Neither knockout group exhibited differences from littermates in sucrose preference suggesting they are also able to form a preference to non-drug rewards. However, cocaine CPP was transient in *A2a-cnr1* and *D1-cnr1* animals and did not persist at the four week abstinence time point. (**Fig 3**). While antagonizing CB1Rs in the NAc is able to inhibit CPP formation, the retention of cocaine CPP may due to CB1R function in the dorsal striatum as the knockouts used in this study do not likely affect NAc MSNs. These results suggest that CB1R function in both neuronal populations is necessary for this retention of cocaine CPP. Lacking CB1Rs in these neurons could increase striatal output in either pathway or alter MSN-MSN inhibitory collaterals, ultimately reshaping striatal circuit function and impacting motivated behavior. These findings suggest that CB1R-dependent regulation of MSN afferents to downstream regions could identify additional targets for treating drug abuse and merits further study.

5.4 Methods

Animals – All animals were bred and housed at Vanderbilt under the supervision of the Department of Animal Care. Conditional cnr1fl/fl mice (Dr. Eric Delpire) were crossed to Drd1a- and Adora2a-cre mice to generate cell-type specific knockouts of CB1Rs. Mice were housed on a 12-hour light/dark cycle and fed ad lib. Breeding cages were given access to 5LOD chow (PicoLab®, 28.7% Protein, 13.4% Fat, 57.9% Carbohydrate) to improve the viability of litters. Upon weaning at P21-28, experimental animals were switched to standard chow.

Behavior – Open field, sensitization, and CPP assays were performed in MedAssociates Activity Test Chambers. All activity was recorded using Noldus Ethovision 9. All mice used in cocaine

experiments underwent a one hour Open Field test to habituate them to the chambers. During habituation, total locomotor activity and center time were monitored.

Sensitization: Mice in sensitization cohorts proceeded to receive intraperitoneal (IP) injections of saline on the following two days before being placed in a homogenous chamber for 20 minutes. On day three through seven, mice were instead given 15mg/kg cocaine IP and similarly allowed to explore the chamber. Cumulative locomotor activity was recorded.

Conditioned Place Preference: Chambers were modified to create two distinct sides based on visual and texture cues. Following habituation, mice were placed into the chamber and allowed to explore freely for 20 minutes to establish an initial preference (pretest). On subsequent conditioning sessions, mice were given a single injection of vehicle (saline) or cocaine (15 mg/kg) IP immediately prior to being placed in the chamber with both sides having the same contextual cues. Side pairings were determined using a biased design with the less preferred side being paired with cocaine. Post-tests were performed following a single pairing and then following three additional pairings. Following the second posttest, mice were housed in home cages for two weeks prior to assessing preference maintenance. Animals in each group were tested at all time points. Locomotor activity was tracked using Noldus Ethovision software.

Sucrose Preference: Mice were separated into single cages for the duration of the assay. Upon initial separation, mice were given access to water from two sipper tubes constructed from a metal drink tube inserted through a rubber stopper placed in a 50mL conical tube. Animals were left in this chamber for 24 hours to habituate to the new environment and drinking apparatuses. Following habituation, water in one of the tubes was substituted for a 2% w/v sucrose solution which the mice were allowed to drink freely from for an additional 24 hours. Preference was assessed by comparing the change in weight of each container over the 24 hour period. Animals had ad lib

access to chow placed on the cage floor for the duration of the experiment.

Drugs – Cocaine hydrochloride was obtained from Sigma Aldrich.

Data Analysis – Electrophysiology experiments were analyzed using using Clampfit 10.4, Microsoft Excel, and Graphpad Prism v6. CPP was analyzed by subtracting time spent on the drug-paired side in pretests from posttest values (ΔCPP). Preference association was determined by comparing raw time on drug paired side to pretest values. Raw time values and ΔCPP were analyzed using two-way repeated measures ANOVA with Sidak's and Dunnet's posttests to examine individual days. For all analyses, alpha was set as 0.05. The number of animals in each group is shown as (n).

CHAPTER 6

Concluding remarks and future directions

The nucleus accumbens and the greater striatal circuitry, along with their inputs, are part of a large neural network that coordinates motivated behaviors. While many modulatory neurotransmitters released in these regions contribute to the shaping of these behaviors, it is theorized that plasticity of glutamatergic transmission mediates the persistence of behavioral adaptations throughout an organism's lifetime. In the previous chapters, the body of knowledge detailing changes in glutamatergic transmission via various signaling cascades with respect to *in vivo* experience has been summarized and expanded upon. These new findings serve to refine current and future models of reward circuit function by clarifying the relationship between plasticity of this circuit to drug-induced behavior. Below, an attempt to place these findings in the broader context of accumbens addiction research is made by delineating what is currently known, what can be gleaned from the above work, and potential topics for future investigation.

6.1 A cohesive picture of mGlu and cannabinoid plasticity in the NAc?

The plasticity of glutamatergic synapses within the NAc has been the subject of intense and ongoing study with respect to the formation of drug-seeking behaviors (See Chapter 2). Metabotropic glutamate receptors (mGlus) in particular occupy a key role in experience, i.e. drug induced, plasticity. mGlus can regulate pre, post, and extra-synaptic function to reshape synaptic transmission onto NAc MSNs. Group I mGlus, expressed postsynaptically in the NAc (Grueter *et al*, 2007), are implicated in exposure to psychostimulants in both contingent and non-contingent assays, yet how they modulate distinct synaptic connections has been relatively unexplored.

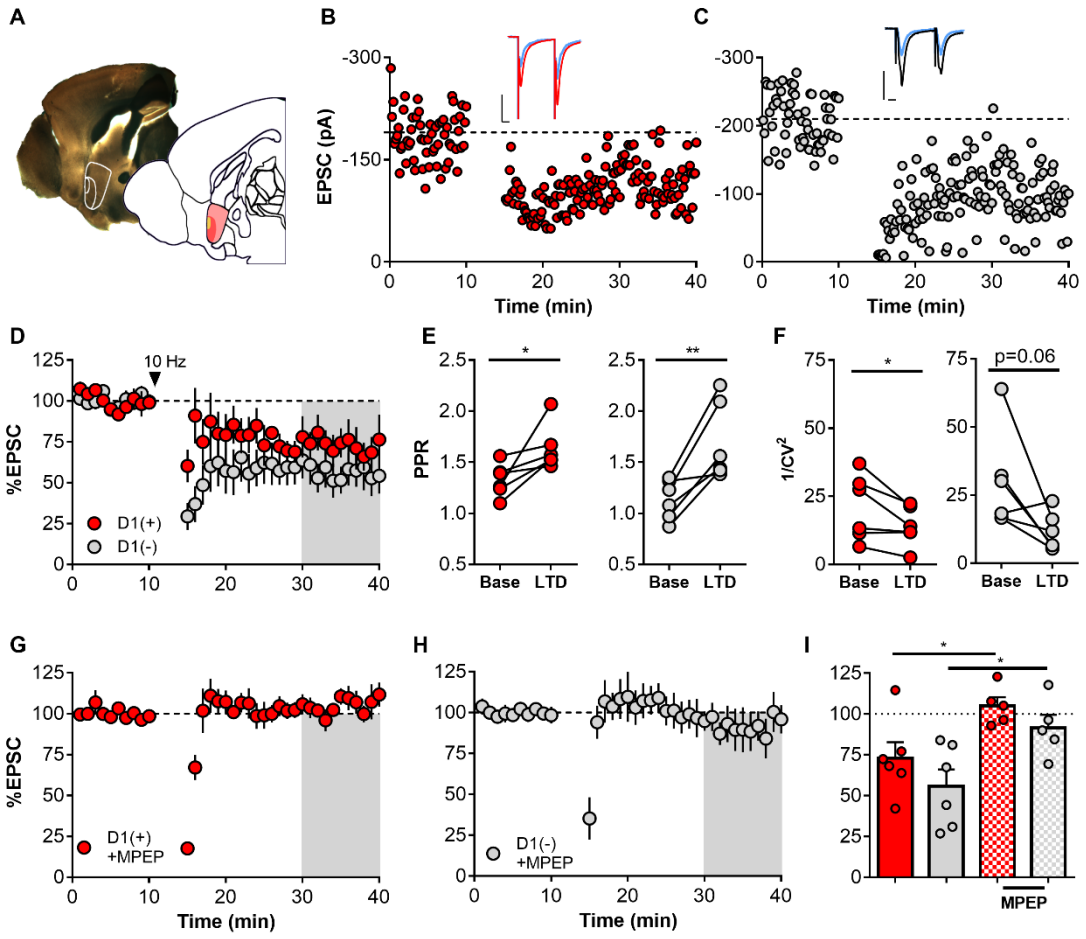


Figure 6.1 - 10Hz stimulation for five minutes is sufficient to induce a robust depression of electrically evoked EPSCs at both D1(+) and D1(-) NAcSh MSNs**. A** Widefield image of a parasaggital mouse brain slice and atlas image highlighting the NAcSh [pink], placement of the stimulating electrode [yellow], and targeted area for whole-cell recordings [red]. **B,C.** Representative experiments and traces from D1(+) [red] and D1(-) [gray] MSNs. Scale: 100 pA/50 ms. **D.** Averaged experiments of 10Hz LTD at D1(+) and D1(-) MSNs. **E** PPR at D1(+) and D1(-) MSNs following LTD induction [D1(+) $p<0.05$, n=6; D1(-) $p<0.05$, n=6, paired t-test]. **F.** 1/CV2 at D1(+) and D1(-) MSNs following LTD induction [D1(+) $p<0.05$; D1(-) $p=0.06$, paired t-test]. **G,H.** Pre-application of 10µM MPEP was sufficient to block LTD induction at both D1(+) and D1(-) MSNs [D1(+) + MPEP, 106.4 ± 6.525, n=5, $p>0.05$; D1(-) + MPEP, 99.97 ± 6.184, n=6, $p>0.05$, one sample t-test vs. 100]. **I.** Summary of LTD induction comparing last five minutes of recording to baseline amplitude [ACSF: D1(+), 73.07 ± 9.681, $p<0.05$, n=6; D1(-), 55.82 ± 10.26, $p<0.05$, n=6, one sample *t* test vs. 100. D1(+) vs D1(+) + MPEP, $p<0.05$; D1(-) vs D1(-) + MPEP, $p<0.05$, student's *t* test].

In the NAc shell, electrical stimulation at 10 Hz for five minutes evokes an mGlu5-dependent LTD at both D1(+) and D1(-) MSNs (**Fig 6.1**). In Chapter 3, it is demonstrated that the same stimulation protocol evoked using optogenetic stimulation induces a Group I mGlu subtype-specific LTD differentially at PFC and MDT synapses with mGlu1 regulating PFC inputs while mGlu5 controls MDT-D1(+) synapses. Knowing that PFC inputs are typically associated with reward-generation while MDT inputs drive aversive behaviors, these findings suggest mGlu1 plasticity would dampen pro-reward PFC inputs while mGlu5 would shift MDT input to favor D1(-) MSN recruitment and promote aversive behaviors. However, it is unfair to assume that this reshaping in synaptic connectivity would result in a shift in MSN firing as both MSNs subtypes specifically those in the dorsal striatum are recruited during the generation of *any* behavioral paradigm (Cui *et al,* 2013) and the same likely holds true for NAc MSNs. However, viewing these patterns *in vivo* is more difficult due to the ventral anatomical location of the NAc. Additionally, inputs from the PFC and MDT may also synapse onto local interneuron population, such as cholinergic (ChAT) and parvalbumin (PV) expressing cells, which exert broad control over NAc circuit function. These synapses may likewise be modulated by mGlu receptors, making it as yet unclear how bulk recruitment of mGlu1 or mGlu5 affects NAc output.

It is also worth noting that the dichotomy normally associated with D1 and D2 MSN activation promoting and opposing reward-association, respectively, is not as clear cut in the accumbens. Several studies have demonstrated that both D1 and D2 activation are associated with reward seeking (Natsubori *et al,* 2017; Soares-Cunha *et al,* 2016a; Soares-Cunha *et al,* 2016b) and that inhibiting or ablating D2 MSNs actually *decreases* motivated behaviors (Tsutsui-Kimura *et al,* 2017). Thus, understanding synaptic function in the NAc alone likely cannot be accurately extrapolated to infer fluctuations in circuit-level activity in various behavioral contexts. More

modern techniques such as *ex vivo* and *in vivo* calcium imaging may be sufficient to bridge this synapse-circuit divide. While calcium transients visualized using fluorescence microscopy do not necessarily coincide with cell firing, this approach is one of the best tools for visualizing whole circuit function in a given context. Indeed, this has already been applied to more dorsal striatal circuits (O'Hare *et al*, 2017) and recently within the NAc as well (Natsubori *et al*, 2017). Future studies can utilize these techniques in tandem with afferent specific stimulation or pharmacological manipulation of receptor systems, such as the mGlu receptors, to determine how these inputs and signaling cascades alter function of NAc MSNs and whether these changes coincide with alterations in motivated behavior.

Despite these caveats, the specificity of Group I mGlu dependent plasticity has interesting implications for mGlu receptor signaling and how it might shape NAc MSN activity during the acquisition of drug-seeking behaviors in current models of reward circuit function. As described in Chapter 2, NAc synaptic plasticity in naïve animals and early phases of drug exposure canonically recruit mGlu5-dependent signaling cascades while prolonged *contingent* exposure shifts plasticity to favor mGlu1 recruitment. mGlu1 PAMs have shown success in ameliorating drug-seeking behavior in such models (Loweth *et al*, 2014a) while mGlu5 PAMs have little effect, though mGlu5 knockout animals are resilient to the generation of drug seeking (Cleva and Olive, 2011; Olive, 2009). Based on the expression of mGlu plasticity in the NAc shell (Chapter 3), the differential recruitment also suggests a shift in relative input strength to the NAc. In naïve animals and early drug-exposure, mGlu5 dominance would lead to enhanced MDT-D1(-) transmission which would favor aversion. This may be why *in vivo* treatment with the mGlu5 PAM VU551 is insufficient to reduce locomotor responding in a cocaine sensitization task (**Fig 6.2**). In late models of drug exposure, the enhanced role of mGlu1, as seen in using non input-specific approaches,

would favor a dampening of PFC inputs potentially reducing the pro-reward seeking behavior engendered by these connections.

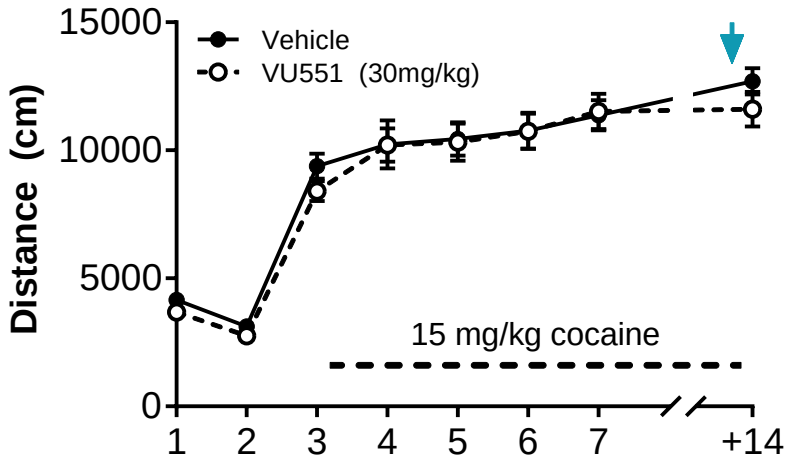


Figure 6.2 - *In vivo* treatment with VU551 is insufficient to reduce the expression of cocaine locomotor sensitization. Intraperitoneal injection of 30 mg/kg VU551 or vehicle (saline) was administered 30 min prior to testing cocaine sensitization (+14). Prior to testing, both groups displayed significant sensitization to cocaine following repeated doses (2-Way ANOVA, Time: $F(7, 98) = 119.2$, $p<0.0001$, Subjects: $F(14, 98) = 6.054$, $p<0.0001$, Interaction, $F(7,98) = 0.5138$, not significant. Vehicle group is significantly different from first day of cocaine at Day 7, Experimental group is significantly different from first day of cocaine after Day 4 [Dunnett's multiple comparisons test]). Groups did not significantly differ on their expression of sensitization following vehicle/drug treatment (Sidak's multiple comparisons test).

In other regions in the striatum, this plasticity protocol (5'10Hz) has been shown to recruit mGlu5-dependent induction of cannabinoid signaling to reduce synaptic transmission via presynaptic CB1Rs and post-synaptic TRPV1. Data presented herein suggests that this plasticity protocol at PFC inputs in the NAc is mechanistically unique as it requires mGlu1 and is not dependent upon CB1Rs (**Fig 6.3**). However, this does not mean that cannabinoid control of these inputs is unimportant. As detailed in Chapter 4, CB1Rs function at PFC terminals in the NAc and CB1R expression in the PFC is required for the generation of CPP to cocaine but not the maintenance of this behavior. At PFC inputs to the NAc, acute exposure to *ex vivo* cocaine seems to recruit eCB signaling at PFC-D1(-) synapses and dampen this signaling at PFC-D1(+) connections, redirecting PFC-NAc signaling to favor D1(+) MSN activation and potentially drive reward association. Lacking CB1Rs at these terminals prevents these physiological changes induced by acute cocaine wash on, suggesting that this shift in PFC-NAc circuitry may be a necessary factor in CPP acquisition.

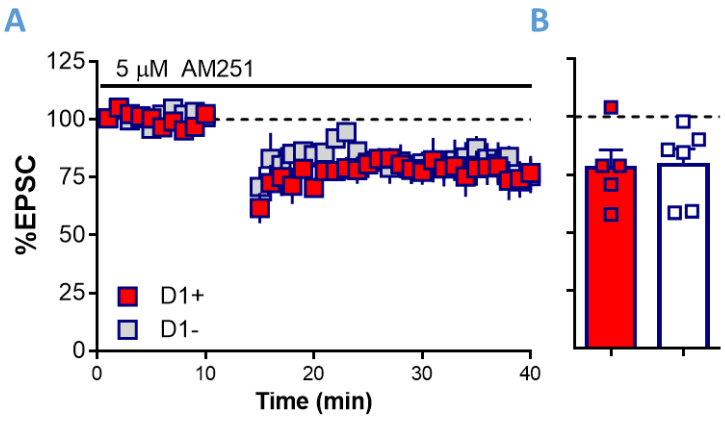


Fig 6.3 - Synaptically evoked LTD of PFC-NAcSh synapses does not recruit CB1Rs.
A. Summary time course of 5'10Hz experiments performed in the presence of the CB1R antagonist AM251. **B.** Change in EPSC amplitude relative to baseline in the presence of AM251 [One sample *t* test: AM251, D1(+) 78.08 ± 7.55 (6), P<0.05, D1(-) 79.35 ± 6.75 (5), P<0.05].

Short term eCB plasticity at PFC-NAc synapses presents a potential point of overlap between the mGlu plasticity and its transition throughout drug-exposure. This is important as PFC-NAc inputs are known to undergo changes in presynaptic release probability following withdrawal from *contingent* cocaine administration (Suska *et al*, 2013), and multiple studies using *in vivo* optogenetics have demonstrated efficacy of this putative mGlu-eCB plasticity mechanism to reduce behavioral metrics associated with drug seeking (Pascoli *et al*, 2014; Pascoli *et al*, 2011). While we do not observe an mGlu-coupled eCB LTD of PFC-NAc synapses, many studies in the NAc have demonstrated that Group I mGlu activation recruits this cascade. It is possible that isolated 10Hz stimulation of PFC inputs alone, while sufficient to activate mGlu1 plasticity, is not sufficient to recruit additional receptor systems that would lead to eCB production. One notable difference is the use of optogenetics to isolate this input while many previous studies have used electrical stimulation of excitatory afferents. While the patch and bath solutions are optimized to visualize glutamatergic (AMPA) currents, electrical stimulation will also recruit non-glutamatergic afferents such as those releasing dopamine and acetylcholine both of which have been shown to contribute to endocannabinoid signaling in other striatal systems (Foster *et al*, 2016; Kreitzer and Malenka, 2005; Lerner and Kreitzer, 2012; Mathur *et al*, 2012).

The apparent involvement of mGlu5-eCB plasticity in NAc with psychostimulant exposure could thus encompass changes at PFC-NAc synapses but this change may also require release of additional neurotransmitters. Future studies could easily address this by examining electrically-evoked plasticity in the NAc while simultaneously isolating PFC inputs to see if local stimulation induces a plasticity via an mGlu5-eCB mechanism. Additionally, one cannot rule out the role of other inputs into the NAc. The differences seen between electrical and optical events is evident in the work detailed in Chapter 4 where *ex vivo* cocaine exposure affects PFC afferents but no change

is observed using electrical stimulation. Similarly, other inputs could underlie the bulk of the mGlu5-eCB plasticity observed using electrical stimulation but these connections do not include PFC afferents and were not sampled in these studies. Thus, future research may focus on the role of mGlu receptors at various other inputs into the NAc in order to create a more complete model with regards to their shaping of MSN synaptic function.

One of the most glaring questions arising from these findings and those by other research groups is how various plasticity cascades are seemingly isolated to synapses corresponding to distinct inputs. While it is easy to conceptualize how CB1R plasticity, or that of any presynaptic signaling cascade, could differ across inputs based on their expression in the afferent region, it is less clear how postsynaptic receptors are regulated in such a manner. Work by Yoland Smith's lab has determined that cortical and thalamic synapses in the NAc express differing levels of mGlu receptors (Mitrano and Smith, 2007). These findings suggest that these receptors are targeted to different synapses based on afferent origin by an as yet unknown mechanism. Co-release of other neurotransmitters from afferent regions could be one way in which these receptors are directed to their respective spines, but this could also be due to signaling through trans-synaptic proteins. These unknown neurotransmitters or trans-synaptic proteins at the synaptic cleft could couple to intracellular cascades that direct transcription and translation to refine the post-synaptic expression of various receptor families thus serving to differentiate these synapses in an afferent-specific manner. These signal transduction pathways and there function at distinct synapses are poorly understood, especially *in vivo*, and provide a rich molecular landscape for further investigation.

6.2 Different inputs, different behaviors

Looking broadly at the reward circuitry, the PFC and the MDT inputs to the NAc play opposing roles in driving behavior not only with regard to valence but also with respect to the

temporal development of reward learning. As seen above, cannabinoid-dependent plasticity of PFC inputs is modified by acute *ex vivo* exposure to cocaine. Additionally, lacking CB1Rs prevents this plasticity and is concurrent with a lack of CPP *formation*. However, knockout of CB1Rs after CPP is already established does not affect the *maintenance* of CPP. Interestingly, inputs from the vSub have been shown to interact with inputs from the PFC, facilitating the transition of MSNs from "Down" (~ -80 mV) to "Up" (~-60 mV) states *in vivo* (O'Donnell and Grace, 1995). Several subsequent studies have demonstrated that bidirectional manipulation of PFC-NAc connections *alone* is sufficient to alter "Up" state transitions in the NAc and striatum (Gruber and O'Donnell, 2009; Kasanetz *et al*, 2006). It is possible that, given the results in Chapter 4, lacking CB1Rs in the PFC disrupts Down-Up state transitions of NAc MSNs and impair their ability to initiate more long-term forms of synaptic plasticity. Additionally, the interaction of PFC and vSub terminals may also explain the lack of CPP formation. Inputs arising from the (vSub) are more commonly attributed with reward-environmental associations (Everitt *et al*, 2005). Thus, one possible explanation for the lack of CPP is a disruption in PFC-gating of MSN state transitions which could impair environment-associated vSub to NAc input from being processed. Due to the likely knockout of CB1Rs at PFC synapses in other downstream targets, the absence of CPP formation cannot be solely attributed to the absence of CB1R signaling at PFC-NAc synapses. However, these results suggest a potential mechanism by which eCBs in the NAc enable a shift in multiple inputs that would coincide with reward association.

Unlike the PFC, inputs from the MDT are implicated in more 'mature' drug-associated behaviors. For example, the Xien lab demonstrated that MDT inputs are strengthened onto D1(-) MSNs following precipitated withdrawal from morphine self-administration and mediate anhedonia-like behaviors associated with said withdrawal (Zhu *et al*, 2016). Additionally,

unpublished works from the Grueter Lab have demonstrated that lacking GluN2B in D1(+) MSNs permits a sustained AMPA receptor dependent enhancement of MDT-D1(+) transmission following abstinence from cocaine in a CPP task. This is correlated with sustained CPP after abstinence from drug whereas wild-type animals lose this preference over time. While the MDT input to the NAc is less understood, these results point to a potential dichotomy where modulation of PFC inputs gate the formation of positive, pro-reward behaviors and plasticity of MDT inputs serve to primarily dampen these behaviors and promote aversion but are recruited during withdrawal and abstinence. Much like examining intracellular and post-synaptic proteins that make these synapses molecularly unique, so too should future experiments examine how these connections interact to drive circuit function at distinct time points in the development of drug and other reward associated behaviors.

6.3 Looking beyond accumbens inputs

Beyond glutamatergic inputs, regulation of inter-MSN connectivity and MSN output also merit further investigation. Chapter 5 entails a brief investigation into how CB1R expression in striatal MSNs regulate cocaine-induced locomotor sensitization and conditioned place preference. Lacking CB1Rs in either D1(+) or D1(-) (Described in the chapter as A2a-*cnr1*) results in a decreased locomotor response to cocaine and impairs cocaine sensitization. D1(+) CB1R null mice displayed a trend towards impaired CPP generation and did not retain the slight preference after prolonged abstinence, while D1(-) CB1R null mice developed a robust preference which was lost following prolonged ‘abstinence.’ While underpowered, current data suggests that knockout of CB1Rs in either cell-type does not impair sucrose preference indicating the mice are not anhedonic and that the mice can form associations with non-drug rewards. Thus, the differences observed relative to wild type littermates may be specific to psychostimulant exposure and suggests that

CB1R-dependent regulation of MSN output is important to the development of drug-associated behaviors. However, MSN output is not limited to downstream projection targets as MSNs also form collaterals that are regulated by CB1Rs (Mathur *et al*, 2013). Additionally, this phenotype is likely not due to changes in NAc physiology, as NAc MSNs do not express CB1Rs (Winters *et al*, 2012). While these findings implicate the dorsal striatum, where D1(+) and A2a(+) MSNs robustly express CB1Rs (Mathur *et al*, 2012; Mathur *et al*, 2013), they highlight the potential impact of MSN output regulation by CB1Rs in shaping drug-related behaviors.

In addition to MSN outputs, interneuron function and the plasticity of their synapses onto NAc MSNs are also poorly understood with respect to the regulation of circuit function during various behavioral tasks. Recent work has identified functional roles for the two main classes of interneurons in the NAc, PV (Qi *et al*, 2016) and ChAT (Brown *et al*, 2012), but these studies only describe bulk activation/inactivation and do not entail synaptic changes. What's more, the modulatory effects of acetylcholine released from ChAT+ interneurons on NAc MSNs are poorly defined with current models of their effects being extrapolated from studies in the dorsal striatum. The NAc microcircuitry encompassing both MSN-MSN collaterals and synapses from local interneurons are promising avenues for future studies of into reward circuit function.

6.4 Not just for drugs: Preliminary studies relating the NAc to hedonic feeding

While the NAc is, as discussed above, well understood for its role in drug abuse and addiction, it is more broadly considered a mediator for all forms of appetitive reward behaviors. Notably, NAc function is also recruited by non-drug rewards including palatable food. As obesity rates rise in Western countries, understanding the role the NAc plays in hedonic feeding presents as a promising avenue for developing therapeutic pharmacological interventions.

Preclinical models of food-seeking behaviors have pointed to glutamatergic transmission

in the NAc as a key mediator of consummatory action. Early work showed that infusion of muscimol, a $GABA_A$ agonist, or CNQX, an AMPA antagonist, directly into the NAc could induce voracious feeding in sated rats (Stratford and Kelley, 1999). This behavior was blocked by also infusing muscimol into the lateral hypothalamus (LH), suggesting a NAc-LH connection contributes to food seeking. More recent optogenetic studies have confirmed this NAc – LH connection is indeed responsible for food seeking, as *in vivo* optogenetic stimulation of D1 terminals in the LH abruptly inhibit licking for sucrose in an operant food self-administration task (O'Connor *et al*, 2015). Of note, these studies, along with others, have focused on the medial accumbens shell, as other regions in the NAc can actually inhibit food seeking when stimulated/inhibited pharmacologically (Richard *et al*, 2013). Regardless, these studies demonstrate that NAc function is essential to food seeking behaviors.

Beyond direct action of glutamate and GABA pharmacology, regulation of NAc function by eCBs is also implicated in food seeking. Acute food-restriction for 24 hours is able to robustly increase concentrations of 2-AG and AEA in the NAc forebrain region. This effect is absent if the animals are able to eat immediately following fasting, suggesting this increase is coupled to pre- and post-prandial states. Additionally, infusion of 2-AG directly into the NAc induces food intake in rats (Kirkham *et al*, 2002). In addition to acute fasting/feeding behaviors, others have shown that dietary manipulations are capable of affecting the NAc eCB signaling system. Omega-3 fatty acid deficient diets fed to pregnant dams inhibited the induction of NAc shell eCB-dependent plasticity in offspring (Lafourcade *et al*, 2011), Acute food deprivation also leads to an increase in CB1R ligand binding (Bello *et al*, 2012), suggesting the cannabinoid system as a whole is in a metabolically replete state. Interestingly, long term exposure to high fat diet decreases CB1R ligand binding in the NAc (Harrold *et al*, 2002), suggesting that diet-induced obesity may likewise

perturb NAc cannabinoid signaling and somehow facilitate a pro-appetitive state. Because of this, we decided to investigate the relationship between metabolic state and NAc eCB signaling using an acute fasting paradigm and short-term exposure to a high-caloric diet.

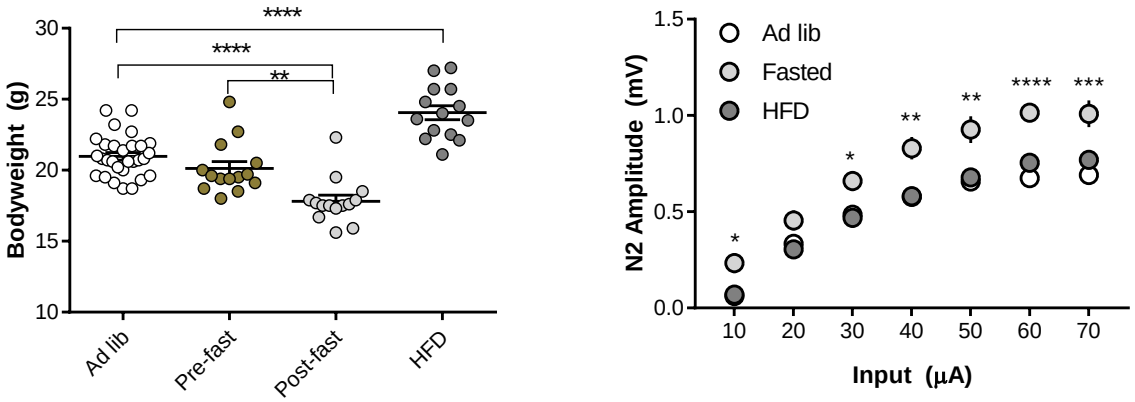


Fig 6.4 - Acute metabolic challenge alters bodyweight and NAc physiology **A.** Bodyweight of animals used in fasting/refeeding physiology and behavior (not shown) [Control: 20.98 ± 0.268; Pre-fast: 20.12 ± 0.489: Post Fast: 17.81 ± 0.431: HFD: 24.05 ± 0.498, all values shown as mean ± SEM. ** = p<0.01, **** = p<0.0001]. **B.** EPSP Input-output relationship is enhanced following acute fasting [*Ad lib*(8), fasted(3), HFD(8); *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, 2-way ANOVA Dunnett's multiple comparison post-test].

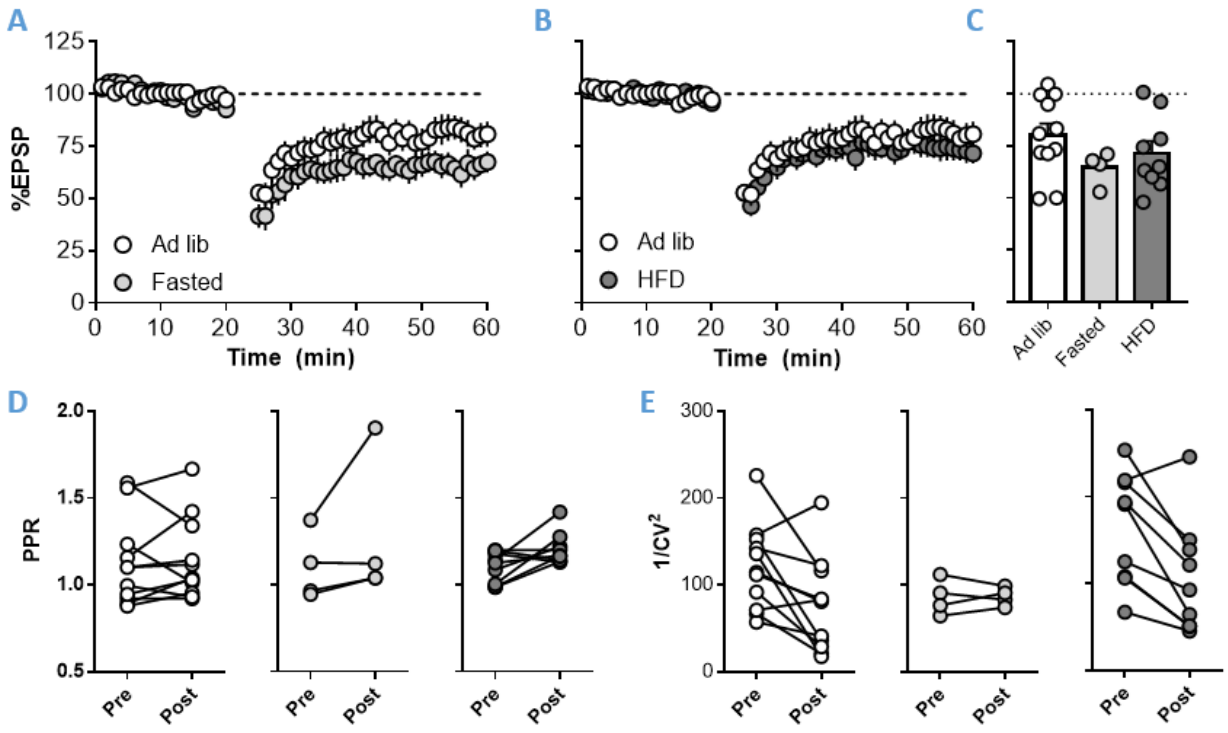


Fig 6.5 - LTD may be enhanced in fasted animals. **A.** 5'10Hz stimulation in extracellular field recordings induces a long-lasting reduction in N2 amplitude in control animals and this effect is enhanced in animals fasted for 18 hours prior to sacrifice. **B.** Post-weaning consumption of HFD for two weeks does not alter the induction of 5'10Hz plasticity in the NAcSh. *Ad lib* in both panel A and B represent the same data. **C.** Summary of plasticity experiments in each group [*Ad* lib: 11, 79.94 ± 5.74 (11); Fasted: 64.56 ± 4.005 (4); HFD: 71.41 ± 5.933(9)]. **D.** Change in PPR following plasticity induction. **E.** Change in $1/CV^2$ following plasticity induction.

In our preliminary studies, we initially investigated how metabolic state affected the putative eCB-dependent plasticity induced by 5' 10Hz stimulation within the NAcSh using extracellular field recordings. As expected, acute fasting resulted in a reduction in bodyweight over 18 hours while acute (2 week) exposure to a high-fat diet resulted in an increase in bodyweight relative to mice fed standard chow *ad lib* (**Fig 6.4**). While exposure to high fat diet did not alter 5' 10Hz plasticity, acute fasting resulted in a larger reduction in excitatory post-synaptic potential (EPSP) amplitude relative to baseline (**Fig 6.5**). This may be due to an increased capacity to undergo long-term depression, as the input-output relationship of stimulation and evoked EPSP amplitude was greater in fasted animals than those given *ad lib* chow or HFD (**Fig 6.4**). These results demonstrated that acute fasting was able to affect plasticity in the NAc shell.

As we saw effects only in acutely fasted animals, we focused future experiments on whether this manipulation altered eCB signaling at specific cell-types in the NAc using whole cell electrophysiology. To measure eCB signaling in a whole-cell configuration, we utilized depolarization induced suppression of excitation (DSE), a canonical method of evoked eCB production (Kano *et al*, 2009; Shonesy *et al*, 2013). While underpowered, preliminary results demonstrate that this protocol results in a CB1R-dependent transient reduction in synaptic transmission (**Fig 6.6, A-C**), and taken together with results shown in Chapter 4 suggest that this protocol does indeed recruit eCBs within the NAc. We chose to use varying times of depolarization to examine a maximal and sub-threshold range of induction intensity to observe either positive or negative deflections in plasticity. Surprisingly, DSE was evoked at both D1 and D1(-) MSNs to an equivalent extent in *ad lib* fed animals regardless of depolarization time (3, 5, and 10 sec @ +30mV, **Fig 6.6, D-E**). Additionally, acute fasting did not affect DSE magnitude at either cell type (**Fig 6.7**). These results are surprising given previous work identifying eCB signaling in the NAc

as a covariant of metabolic state but they do not rule out more nuanced changes that may be unobservable using the aforementioned approaches.

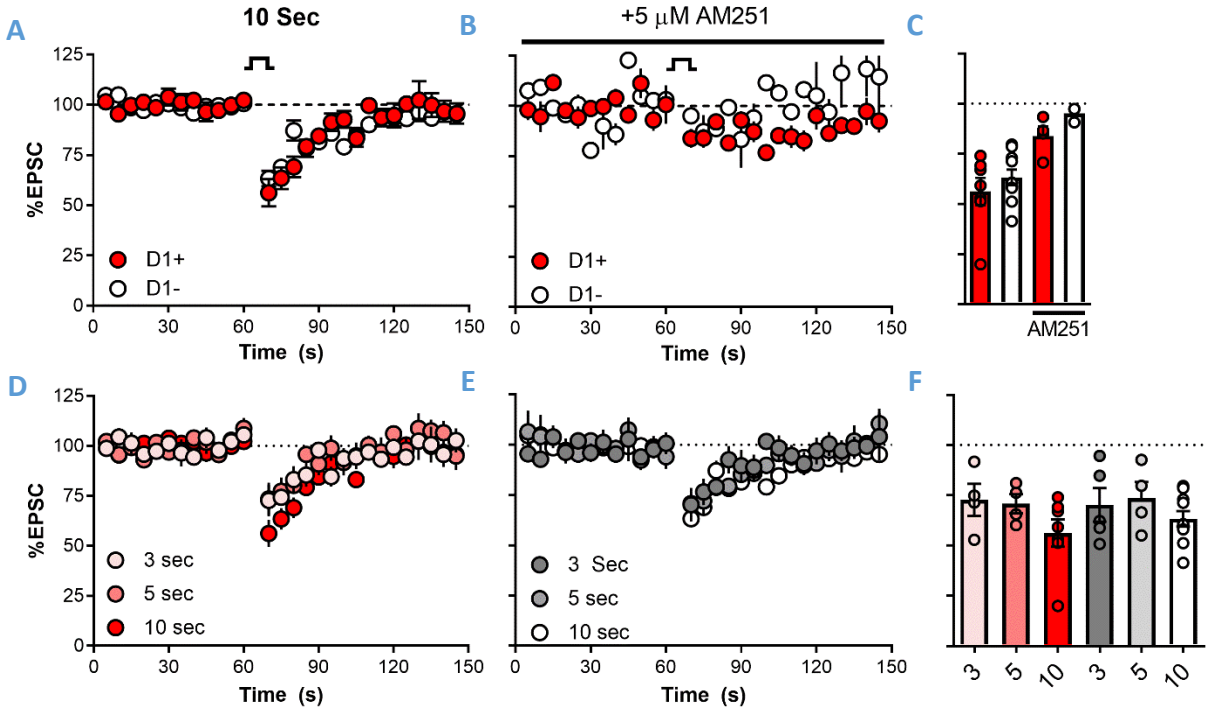


Fig 6.6 - DSE is reduced by AM251 but is not dependent on time spent depolarized. **A.** Timecourse of DSE evoked at D1+ MSNs with or without AM251 (10 μM). **B.** Time course of DSE evoked at D1- MSNs with or without AM251. **C.** Average first EPSC evoked following depolarization (gray bar) in each DSE experiment with or without AM251 [D1+ no drug, 56.23 ± 6.826(7), AM251 83.95 ± 4.781(4); D1- no drug, 63.3 ± 3.867(10), AM251 95.15 ± 2.221(3)]. **D,E.** Time course of reduction in EPSC amplitude following 3, 5, and 10 second depolarization to +30 mV, respectively. **F.** Reduction in EPSC amplitude as a percent of baseline for each depolarization protocol represented as an average of the first event sampled following return to -70mV [D1+, 3sec 72.72 ± 7.964(4), 5sec 73.5 ± 8.142(4); 10sec 56.23 ± 6.826(7). D1-, 3sec 70.27 ± 8.301(5), 5sec 70.76 ± 4.807(4), 10sec 63.3 ± 3.867(10), values shown as mean ± SEM(*n*)].

For example, as seen in Chapter 4, changes induced by cocaine *ex vivo* are observable only at PFC synapses in the NAc core while electrically evoked events remain similar in both treated and untreated conditions. Such findings suggest that the negative results obtained above may simply reflect the inability to observe specific changes in eCB signaling at a subset of synaptic connections within the NAc circuitry. Notably, only the MDT input into the NAc has been implicated directly in food-seeking behaviors (Do-Monte *et al*, 2017), while others, including the PFC (Pascoli *et al*, 2014), have been specifically ruled out and seem to engender behavioral responding to only psychostimulants in the experimenter's models. While it is unlikely that PFC inputs are restricted to drug-related behavior, these results as a whole imply that separate circuits overlapping in the NAc regulate food and drug behaviors. This is somewhat unsurprising knowing that, despite its known role in the generation of drug-seeking behaviors, the NAc circuit responds differently to various abused substances (Joffe *et al*, 2016; Neumann *et al*, 2016; Turner *et al*, 2018; Zhu *et al*, 2016) and that different classes of drugs do not cross-sensitize in a coherent manner (Joffe *et al*, 2014). Additionally, one cannot discount temporal differences in these studies with regards to sample preparation. In the abovementioned studies examining eCB function and food intake, samples were flash-frozen or otherwise preserved immediately after sacrifice of the animal. In our preparations for physiology, slices are left to incubate for at least one hour at room temperature in ACSF. It is possible that any changes seen in cannabinoid species concentration is limited to the time immediately after sacrifice and that prolonged incubation thereafter allows for sufficient degradation of the small molecules. This is pertinent if the changes expected do not involve long-term modification of eCB production or degradation pathways.

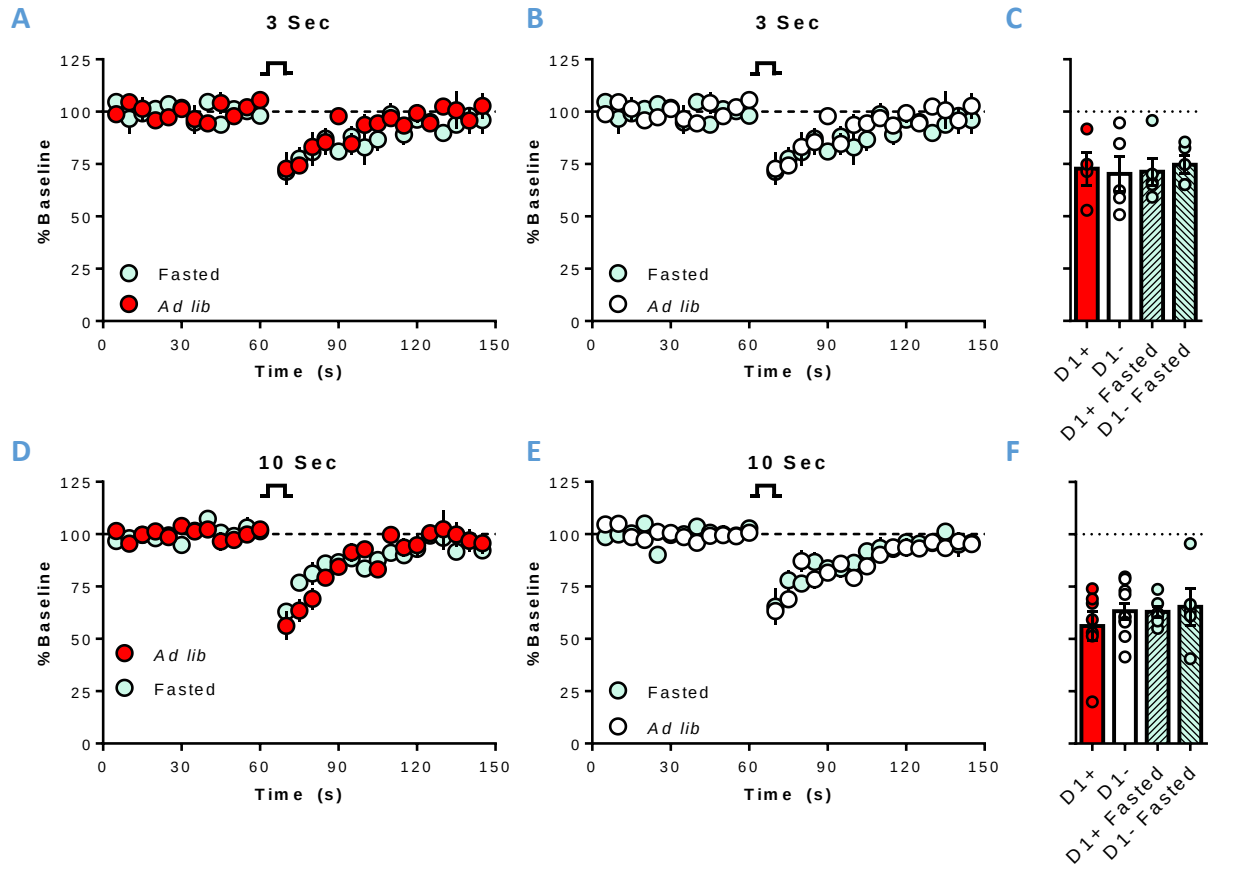


Fig 6.7 - Fasting does not alter DSE amplitude evoked at NAc MSNs **A,B.** Time course of DSE induced using 3sec depolarization at D1(+) and D1(-) MSNs from *ad lib* and fasted mice. **C.** Summary of 3sec DSE induction [3sec fasted: D1(+) 71.28 ± 6.372 (5), D1(-) 74.65 ± 4.254 (5), values shown as mean ± SEM (*n*)]. **D,E.** Time course of DSE induced using 10sec depolarization at D1(+) and D1(-) MSNs from *ad lib* and fasted mice. **F.** Summary of 10sec DSE induction [10sec fasted: D1(+) 62.93 ± 2.7 (6), D1(-) 65.39 ± 8.808 (5)]. Note: Data and statistics from *ad lib* fed mice is also shown in Fig 6.6.

It should be said that it is perhaps unwise to superimpose the widespread knowledgebase of drug addiction onto food seeking behaviors ((DiLeone *et al,* 2012) given the fact that the two seem to recruit distinct circuits. However, similar changes observed in drug-abuse models are also observed in food self-administration paradigms. Notably, like the increase in AMPA transmission seen in psychostimulant models, acute withdrawal from palatable high-caloric diet is able to induce the expression of calcium-permeable AMPA receptors, albeit at a much earlier time point (Oginsky *et al*, 2016). Given these overlapping physiological findings and their potential therapeutic impacts, it is the hope of this author that future investigations surrounding 'natural' rewards will be undertaken in regards to reward circuit function.

6.5 Closing

Above, I have hopefully disseminated a succinct and summarized version of my work as well as its implications for future researchers. While all scientific studies are almost, by definition, incomplete, it is my hope that this body of work is sufficient to further the study of motivation and reward as it relates to the NAc both within the academic environment at Vanderbilt and beyond. The NAc circuitry and that of the greater reward circuitry has been implicated in multitudinous disorders and behavioral paradigms time and time again and undoubtedly will serve as the focus of future research to come.

6.6 Methods

Animals - All animals were bred and housed at Vanderbilt under the supervision of the Department of Animal Care. Transgenic BAC *Drd1a-tdTomato* mice were obtained from JAX laboratories and bred to C57BL/6J wild type females. Conditional *cnr1*$^{fl/fl}$ mice (Dr. Eric Delpire) were crossed to *B6;129S-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J* and BAC Drd1a-tdTomato mice to obtain the triple transgenic *cnr1*$^{fl/fl}$*-chr2*$^{fl/fl}$*-tdTom* mouse line. Animals were back-crossed for six generations prior to use in experiments. DC/AC x CNR1 - All animals were housed on a 12-hour light/dark cycle and fed *ad lib*. Breeding cages were given access to 5LOD chow (PicoLab®, 28.7% Protein, 13.4% Fat, 57.9% Carbohydrate) to improve the viability of litters. Upon weaning at P21-28, experimental animals were switched to standard chow.

Metabolic manipulation – Fasting: Animals were food restricted in pairs by transferring them to clean cages lacking food. Animals had access to a water bottle for the duration of the fast which was initiated near the beginning of the dark cycle. Animals were weighed before and after placement in the fasting cages.HFD – Animals were given ad lib access to OpenSource ® D12492 high fat chow (20% protein, 20% carbohydrate, 60% fat by kcal) for at least two weeks following weaning.

Whole-cell electrophysiology - Mice were anesthetized using isoflurane prior to sacrifice. Parasaggital sections (250μm) containing the NAcSh were prepared from whole brain tissue using a Leica Vibratome. Slices were briefly placed in an N-methyl D-glucamine (NMDG) based recovery solution (2.5 KCL, 20 HEPES, 1.2 NaH_2PO_4, 25 Glucose, 93 N-methyl-D-glucamine, 30 $NaHCO_3$, 5.0 sodium ascorbate, 3.0 sodium pyruvate, 10 $MgCl_2$, and 0.5 mM $CaCl_2$-$2H_2O$) for 10-15 min at 32°C before transfer to a chamber containing artificial cerebral spinal fluid (ACSF, 119 NaCl, 2.5 KCL, 1.3 $MgCl_2$-$6H_2O$, 2.5 $CaCl_2$-2HO, 1.0 NaH2PO4-H2O, 26.2 $NaHCO_3$, and

11 mM glucose) until use. All electrophysiology experiments were performed using a Scientifica Slicescope Pro System under a constant perfusion of 32°C ACSF at a rate of 2 mL/min. NAcSh MSNs were visualized with a Scientifica PatchVision software and patched with 3-5 MΩ recording pipettes (P1000 Micropipette Puller) filled with a cesium-based internal solution (120 $CsMeSO_3$, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4.0 Mg^2-ATP, 0.3 Na^2-GTP, 0.1 spermine, and 5.0 mM QX 314 bromide) in LTD experiments (Chapter 2) and AMPA/NMDA ratio experiments (Chapter 3). For DSE experiments, and all experiments referenced in Chapter 3, the internal solution consisted of 125 mM, 4 NaCl, 10 HEPES, 4 Mg2+ATP, 0.3 Na+GTP, and 10 mM Na+ phosphocreatine.

MSNs were identified based on visual appearance (size, morphology) as well as electrophysiological properties (membrane resistance, capacitance, and the presence of currents at +40 mV to exclude fast-spiking interneurons); D1 MSNs were identified by fluorescence of tdTom. All experiments were performed in the presence of the $GABA_A$ channel blocker picrotoxin (50μM). Experimental protocol execution, stimulation control, and data collection were performed using a Molecular Devices pClamp 10 Analysis software. Control and monitoring of cell electrical properties were achieved using an Axopatch 500B Multiclamp amplifier and Axon Digidata 1550 low-noise data acquisition digitizer. Responses were filtered at 2 kHz and digitized at 10 kHz. Optical stimulation of ChR2-expressing terminals was achieved using a CoolLED pE-100 LED excitation system. 480 nm light was pulsed through the high-powered (40x) objective to excite ChR2+ terminals at 0.1 Hz for 0.5-1 ms. Light intensity was adjusted to evoke stable responses.

Paired stimuli were delivered at 20, 50, 100, 200, and 400 ms to generate the paired pulse ratio curves. Depolarization induced suppression of excitation (DSE) was evoked by depolarizing patched MSNs to +30mV for 10 seconds. For cocaine wash-on experiments, Cocaine-HCl was

dissolved in H_2O at 20mM and diluted to 10μM in ACSF. Stock solutions were prepared fresh weekly. Cocaine was washed on for 10 minutes while recording. PPR and DSE experiments were performed at least 10 minutes following removal of drug from the bath solution. NMDA currents in AMPA/NMDA ratios were determined by the peak amplitude 50ms post onset of the +40mV EPSC.

Extracellular field recordings – Borosillicate extracellular recording pipettes (1-2 MΩ) were filled with ACSF (see above) and pierced slightly into the tissue of the NAcSh approximately 100 um ventral-caudal from the stimulating electrode. An input-output relationship was determined for each set of experiments, stimulating the tissue at 10 – 100 uA intensity range with 10 uA intervals prior to long term recording. A median I/O intensity was chosen for LTD experiments.

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