NUCLEUS ACCUMBENS N-METHYL-D-ASPARTATE RECEPTOR FUNCTION AND REWARD LEARNING: IMPLICATIONS FOR COCAINE USE DISORDERS

By

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To Milton H. Joffe and his great-grandchildren.

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CHAPTER I

INTRODUCTION

1. Background

Addiction is a devastating disease that imposes a substantial toll on afflicted individuals, close friends and relatives, and society as a whole. According to the American Society of Addiction Medicine, addiction is a primary, chronic disease of brain reward, motivation, memory, and related circuitry. An important characteristic of addiction is the inability of a patient to consistently abstain from addiction-related behavior despite negative consequences to himself and those around him. Each year in the United States, abuse of illicit drugs alone has been estimated to exact over $11 billion in direct health care costs and over $193 billion related to social and occupational factors (National Drug Intelligence Center, 2011). Moreover the disease is pervasive and affects all regions, socioeconomic classes and ethnicities. In the Nashville Metro Area the average annual prevalence of a substance use disorder diagnosis has been estimated to be 9.3% (Figure 1, Page 2) (SAMHSA, 2012).

Substance use disorders involve the persistent abuse of psychoactive drugs, small molecules that affect neuronal function by altering the biochemical balance of the brain. Exposure to these agents results in alterations of behavior, consciousness and mood; such agents include alcohol, tobacco, cannabinoids, opioids, stimulants, hallucinogens, club drugs and some prescription drugs. Non-

drug addictions have also been described including sex, gambling, and other behaviors. Likewise the concept of overindulging in consumption of highly palatable foods has been recently debated as constituting an addiction. This should not be surprising, since drugs of abuse hijack the natural reward circuitry which has been evolutionarily optimized for survival (DiLeone et al., 2012).

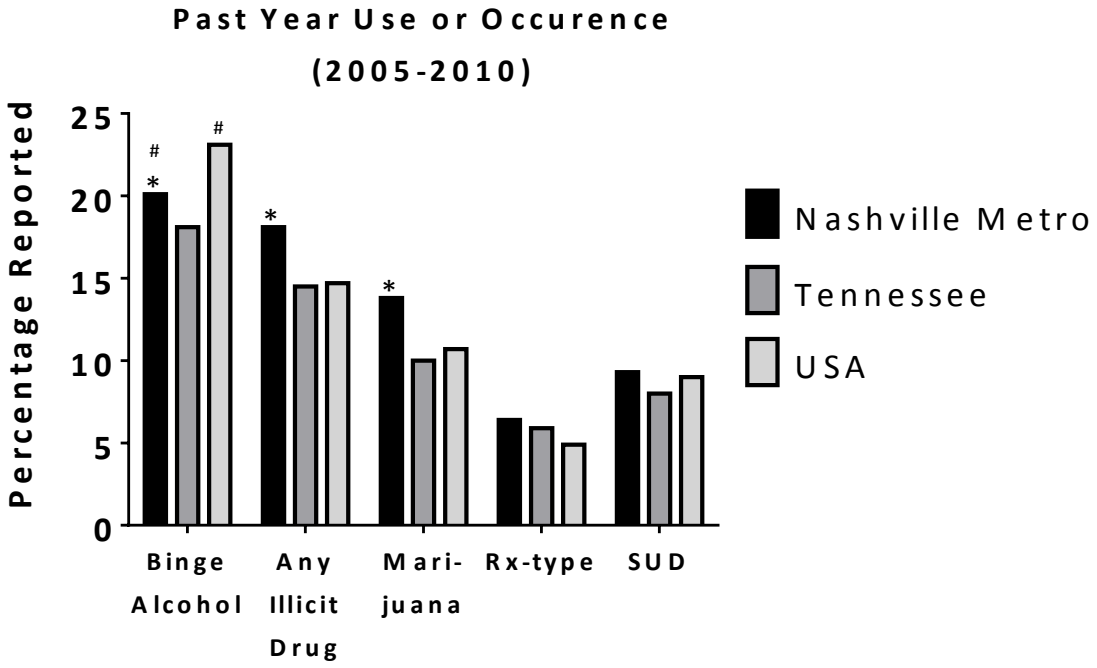


Figure 1. Annual prevalence of illicit drug use and substance use disorder. Data adapted from 2012 National Survey on Drug Use and Health Report. Nashville Metro refers to the Nashville, Davidson County, Murfreesboro, and Franklin. Rx-type refers to painkillers. SUD, substance use disorder. USA, United States of America. (*: $p < 0.05$ vs Tennessee, #: $p < 0.05$ vs. USA)

More specifically, addiction is a chronic disease characterized by a cyclical, relapse-laden progression through several phases of maladaptive behavior (Koob and Volkow, 2010). The cyclical nature of the disease is particularly evident with substance abuse patients, who repeatedly progress through periods of binging (high levels of consumption), withdrawal (abstinence, in the presence of anxiety

and/or negative affect), and preoccupation (intense craving and anticipation of next use). Consummatory behavior at disease onset is generally impulsive, and thought to be motivated mainly by positive reinforcement (i.e. euphoria obtained from substance ingestion). However as the disease progresses, the motivation underlying drug-seeking shifts towards negative reinforcement (i.e. relief of withdrawal symptoms). Eventually, the drug-taking is maintained by compulsive habitual behaviors. While all drugs of abuse act on a final common pathway, the sensitization and intensification of these symptoms and phases is thought to recruit distinct neural circuits and local networks. Thus, to an extent, it is possible to map the phases of addiction-related behaviors onto the function of specific neural circuits.

1.1 Neuropharmacology of Abused Substances

To understand how abused substances cause durable changes in the brain, it is first necessary to introduce how they acutely alter brain function (reviewed by Sulzer, 2011). Drugs of abuse elicit their effects through a variety of mechanisms. Yet all abused substances recruit the major natural reward pathway of the central nervous system, the mesocorticolimbic dopamine system. All drugs of abuse directly or indirectly facilitate the release of the neurotransmitter dopamine, produced in the ventral tegmental area (VTA) of the midbrain, in the nucleus accumbens (NAc) (Di Chiara et al., 2004) (Figure 2, page 5). The receptor targets for drugs of abuse have been well defined and most are cell surface receptors, however understanding their complex downstream effects remains a challenge

and these are described in a subsequent section. Here we will briefly describe the targets for well-studied drugs of abuse (Table 1).

Table 1

Drug(s) of Abuse	Primary Action	Physiological Effects
cocaine, methylphenidate, MDPV derivatives	DAT inhibition	Increased blood pressure and heart rate, increased energy and alertness, reduced appetite, anxiety, paranoia, psychosis
amphetamine and cathinone derivatives	VMAT inhibition	
nicotine	nAchR agonism	Increased blood pressure, heart rate, and alertness
heroin, morphine, and synthetic analogs	MOR agonism	Euphoria, drowsiness/sedation, nausea, respiratory depression
***Salvia divinorum* / salvinorin A**	KOR agonism	Dissociation, hallucinations, impaired motor function
PCP, ketamine	NMDAR antagonism	Dissociation, delirium, analgesia, impaired motor function
Barbiturates, benzodiazepines, Z-drugs	$GABA_AR$ positive modulation	Sedation, anxiolysis, amnesia, impaired coordination, muscle relaxation
***Cannabis*, THC, and analogs**	CB_1R agonism	Altered perception, impaired memory, increased heart rate and appetite
Tryptamines/phenethylamines (e.g. psilocin, mescaline, LSD, DOM, DMT)	$5\text{-}HT_{2A}R$ agonism	Altered perception, hallucinations, emotional changes, insomnia
Alcohol (ethanol)	pleiotropic effects	Relaxation, loss of inhibition, drowsiness, impaired coordination, amnesia
Solvents and inhalants	pleiotropic effects	Stimulation, slurred speech, impaired coordination

Table 1. Common Drugs of Abuse. Drugs of abuse, their primary mechanism of action, and commonly observed physiological effects. Despite the diversity of primary mechanism of action, all drugs of abuse facilitate dopamine release in the nucleus accumbens. $5HT_{2A}R$, serotonin receptor subtype 2A; CB_1R, cannabinoid receptor subtype 1; DAT, dopamine transporter; DMT, *N,N*-dimethyltryptamine; DOM, 2,5-dimethoxy-4-methylamphetamine; $GABA_AR$, γ-aminobutyric acid receptor subtype A; KOR, kappa opioid receptor; LSD, lysergic acid diethylamide; MOR, mu opioid receptor; nAchR, nicotinic acetylcholine receptor; NMDAR, N-methyl-D-aspartate glutamate receptor; PCP, phencyclidine; THC, tetrahydrocannabinol; VMAT, vesicular membrane monoamine transporter.

Psychostimulants, including cocaine and amphetamines, act directly on the presynaptic dopamine-releasing axon terminals of VTA afferents. Cocaine impedes the clearance of dopamine from the synaptic cleft by inhibiting plasma membrane monoamine transporters, whereas amphetamines alter the function of both the vesicular monoamine transporters and plasma membrane transporters (Robertson et al., 2009; Sulzer et al., 2005). Nicotine binds to the orthosteric binding site of the nicotinic acetylcholine-gated cation channel (nAChR). Nicotine is thought to act primarily through activation of α4β2-containing nAChRs on dopamine cell bodies and axon terminals, thereby directly leading to dopamine release in the NAc (Xi et al., 2009). However, nicotine-mediated activation of α7 homomers on dopamine axon terminals and presynaptic glutamatergic afferents may too be involved.

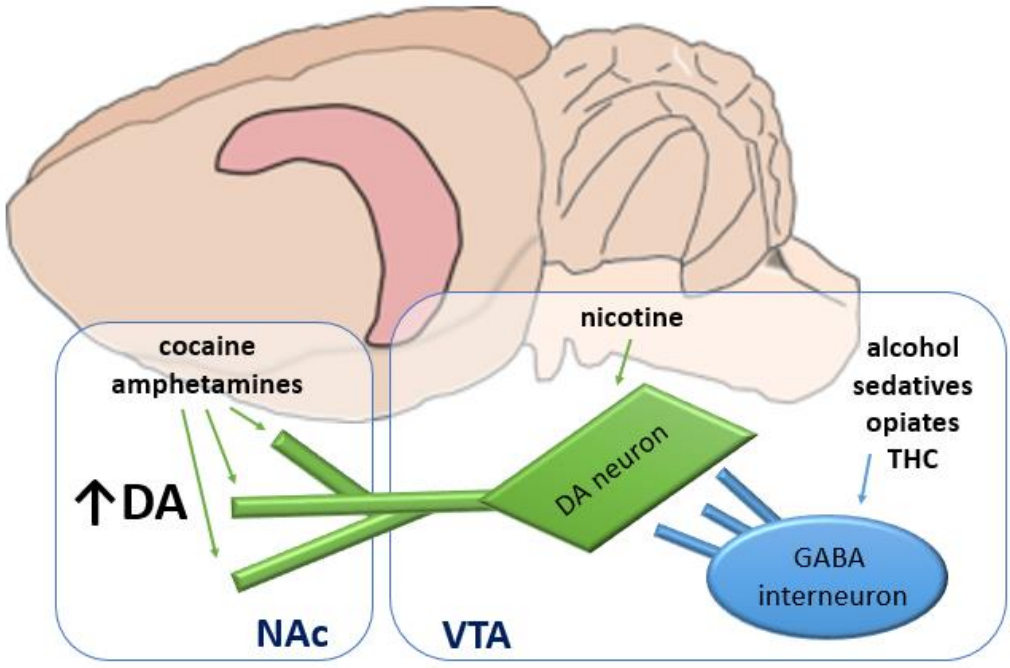


Figure 2. All drugs of abuse facilitate DA release in the NAc. Despite heterogeneous molecular and cellular targets, all drugs of abuse increase extracellular DA concentrations in the NAc. DA, dopamine; GABA, γ-aminobutyric acid; NAc, nucleus accumbens; THC, tetrahydrocannabinol; VTA, ventral tegmental area. Image adapted from Lüscher and Malenka, 2011.

Opiates like heroin and morphine activate $G_{i/o}$-coupled μ-opioid receptors (MOR), which, within the VTA, are expressed mainly on GABAergic interneurons. Activation of MORs hyperpolarizes VTA interneurons, leading to disinhibition of the dopamine projection neurons and enhanced dopamine release in the NAc (Ting-A-Kee and van der Kooy, 2012). Similarly, several other classes of abused drugs – barbiturates/benzodiazepines, PCP/ketamine, and cannabinoids – are thought to facilitate dopamine release in the NAc via similar mechanisms (Lüscher and Ungless, 2006). Like other non-stimulant drugs of abuse, ethanol causes a net disinhibition of VTA dopaminergic neurons (Marty and Spigelman, 2012), however ethanol has a rich pharmacology involving direct interactions with glutamatergic and GABAergic ion channels.

1.2 Neurocircuitry of Reward

Seminal work from Olds and colleagues demonstrated that rodents will work to electrically stimulate relatively discrete areas of the brain (Olds and Milner, 1954). Considering the observation that humans find stimulation of these same areas pleasurable, these regions were described as comprising the brain-reward circuitry (Wise, 1996). Subsequently, others have shown that animals will work to self-administer drugs of abuse (but not other drugs) and that this self-administration behavior is disrupted by lesioning brain-reward regions (Koob et al., 1998) (Figure 3, page 7).

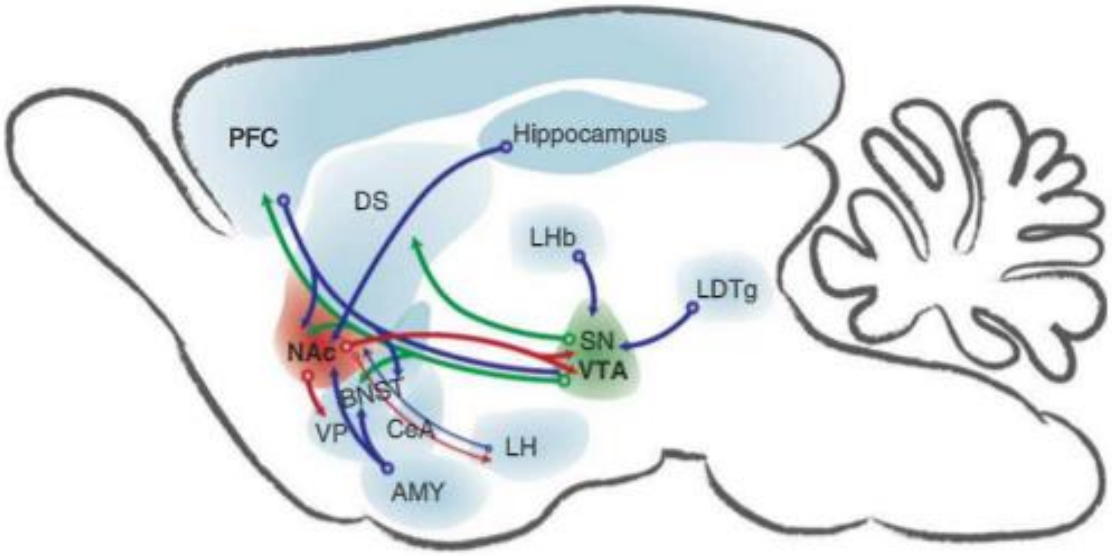


Figure 3. Simplified schematic of the reward circuitry in the rodent brain emphasizing signaling to and from the nucleus accumbens (NAc) and ventral tegmental area (VTA). Glutamatergic transmission drives information through the reward and reward-related circuitry (blue arrows). GABAergic transmission from NAc and other regions dampens target neuronal activity (red arrows). Dopamine release from the VTA and substantia nigra (SN) modulates synaptic transmission in target regions (green arrows). These regions are recruited and undergo synaptic, circuit, and genetic adaptations in response to drug experience. AMY, amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; DS, dorsal striatum; LDTg, laterodorsal tegmentum; LHb, lateral habenula; LH, lateral hypothalamus; PFC, prefrontal cortex; SN, substantia nigra; VP, ventral pallidum.

The critical regions in the reward circuitry are now widely accepted to include the mesolimbic dopamine system, more specifically the VTA-NAc pathways (Kalivas et al., 2005). Yet, the VTA-NAc pathways comprise only part of a series of parallel, integrated circuits, which involve several other key brain regions. These other regions include but are not limited to the prefrontal cortex (PFC), midline nuclei of the thalamus (mThal), ventral hippocampus (vHipp), and basolateral amygdala (BLA) which all provide excitatory drive within the reward circuit. Auxiliary regions, including the dorsal striatum and extended amygdala, sustain habitual behaviors and stress responses in addiction.

1.2.1 Dopamine Centers

Dopamine plays a central role in motivation and reward processing. However, dopamine-deficient mice still demonstrate a degree of reward learning, suggesting the monoamine is not necessary for this process (Wise, 2009). These findings illustrate that although dopamine is key to reward, the holistic reward system is substantially more complex. Dopamine production occurs in midbrain dopamine neurons including the VTA, the retrorubral field and the substantia nigra (SN). In addition to dopamine neurons, the VTA contains GABAergic neurons, which can regulate VTA dopamine neuron function (Tan et al., 2010), and/or project to and modulate NAc cholinergic interneurons (Brown et al., 2012). In addition to having widespread projections throughout the brain, retrograde tracing techniques have shown that the VTA receives diverse afferent inputs, which likely influence behavioral output differentially (reviewed in Lammel et al., 2013).

The SN is a midbrain dopaminergic region closely related to the VTA. Like the VTA, electrical stimulation of the SN is rewarding. Additional evidence suggests that blockade of glutamatergic or cholinergic signaling into either the SN or VTA alters addiction-related habit formation (Wise, 2009). Also, blockade of dopamine receptor signaling in the terminal beds of either the VTA (NAc) or the SN (dorsal striatum) alters addiction-related behaviors, although to a much greater degree in the NAc. Utilization of optogenetics and genetic engineering in rats and mice has allowed for more detailed analysis of addiction-related behaviors as the light sensitive channels that activate (channelrhodopsins) or inhibit (halorhodopsins) cellular excitability can be expressed in a region- and cell type-

specific manner. For instance, selective photostimulation and subsequent excitation of VTA dopamine neurons supports reward related behaviors such as conditioned place preference (Tsai et al., 2009; Witten et al., 2011).

Like the SN, the VTA also degenerates during the progression of Parkinson's disease (PD) and may explain some of the similarities between the PD non-motor symptoms and post-acute-withdrawal syndrome (e.g. depression/irritability, cognitive disruptions). Overall, it is well accepted that the activity of midbrain dopamine neurons conveys information involved in integration of the rewarding vs. aversive properties of environmental stimuli (Lammel et al., 2013).

1.2.2 Dorsal Striatum and Nucleus Accumbens

The striatum is the gate to the basal ganglia, integrating inputs from the PFC, as well as thalamic and limbic structures (vHipp and BLA). Consisting of multiple nuclei differentiated by their anatomical connections and behavioral functions, the striatum is broadly divided into a dorsal region, also known as the caudate-putamen, and a ventral region, which mainly consists of the NAc. The NAc is further divided into two subregions, the core and the shell with the core being more similar to the dorsal striatum and the shell having strong similarities with the extended amygdala (described below). Serial connectivity between the NAc, midbrain, and dorsal striatum may account for the transition from motivated to habitual behaviors observed in the progression of addiction (Belin and Everitt, 2008; Haber et al., 2000). Thus, drug-evoked changes in synaptic strength and

connectivity within the dorsal striatum and the NAc are thought to underlie many behavioral components of addiction (Grueter et al., 2012).

The known neuronal cell types in the dorsal striatum and NAc include GABAergic projection medium spiny neurons (MSNs), multiple types of GABAergic interneurons, and cholinergic interneurons. The MSNs can be further subdivided into two categories (historically referred to as “direct pathway” (striatonigral) and “indirect pathway” (striatopallidal) MSNs) based on projection targets, electrophysiology, and expression of neuropeptides and cell surface receptors (Figure 4). For instance, dopamine receptor subtype 1 (D1R), muscarinic acetylcholine receptor subtype 4 and prodynorphin are expressed by one MSN type whereas the other type expresses dopamine receptor subtype 2 (D2R), adenosine receptor subtype 2A (A2AR) and proenkephalin.

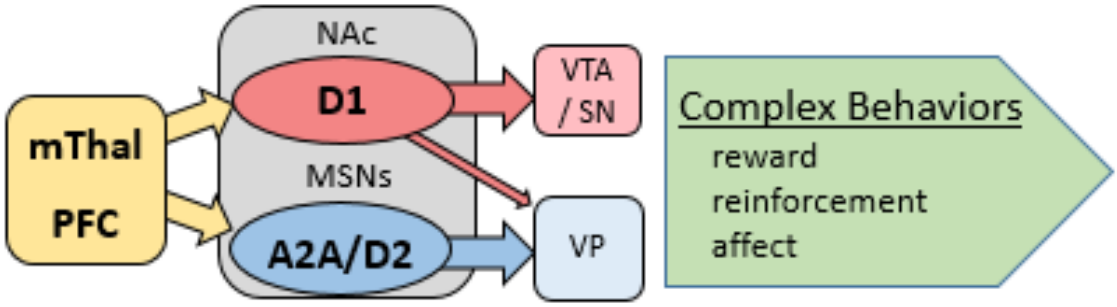


Figure 4. Simplified schematic of NAc circuity. NAc MSNs are readily divided into two groups. One group expresses D1 and projects to the midbrain (and VP) whereas the other expresses A2A and D2 and projects to the VP. While some overlap exists with regards to protein expression and anatomy, the balance of MSN pathway function is hypothesized to regulate several complex behaviors related to motivational state. Because MSNs are quiet and generally rest at hyperpolarized potentials, excitatory drive is crucial in driving MSN activity *in vivo* and modulating NAc-related behavioral outcomes. A2A, adenosine receptor subtype 2A; D1, dopamine receptor subtype 1; D2, dopamine receptor subtype 2; MSN, medium spiny neuron; mThal, midline nuclei of the thalamus; NAc, nucleus accumbens; PFC, prefrontal cortex; SN, substantia nigra; VP, ventral pallidum; VTA, ventral tegmental area.

D1 MSNs mainly project to dopaminergic midbrain regions, whereas D2 MSNs primarily target the pallidum (Smith et al., 2013), although these distinctions are far from clear, especially in the NAc shell (Kupchik et al., 2015). Several studies have demonstrated that these parallel circuits can exert opposing or dichotomous functional effects on behavior (Francis et al., 2014; Grueter et al., 2013; Kravitz et al., 2012; Lobo et al., 2010), whereas others suggest that the pathways may work in tandem towards the same functional response (Beutler et al., 2011; Cui et al., 2013).

Excitatory (glutamatergic) drive is critical for MSN output as these neurons are relatively quiescent with a resting membrane potential around -80 mV. The dorsal striatum receives excitatory inputs primarily from the associative and sensorimotor cortex and thalamic nuclei, as well as reciprocal dopaminergic innervation from the SN. In contrast, the NAc receives excitatory inputs from the prefrontal cortex and limbic regions. Although both the NAc shell and core receive inputs from the VTA, the NAc shell sends reciprocal projections to the VTA while the NAc core projects back to the SN. An essential role for the NAc in drug-related behaviors is evidenced by the demonstration that psychostimulant self-administration is disrupted by NAc-specific lesions, or local blockade of glutamate or dopamine receptors.

In addition to its well-characterized role in the rewarding and reinforcing properties of drugs of abuse and natural stimuli, the NAc has also been implicated in the placebo effect (Scott et al., 2007) and the processing of pleasant emotions induced by imagery (Costa et al., 2010) and music (Menon and Levitin, 2005). By

contrast overactive or otherwise dysfunctional activity in the mesolimbic system is thought to be involved in the manifestation of positive psychotic symptoms (e.g. delusions, hallucinations, grandiosity) shared by acute stimulant-induced psychosis and psychiatric illnesses like schizophrenia and bipolar disorder.

1.2.3 Glutamatergic Regions of the Reward Circuit

The PFC, often divided in rodents into the prelimbic and infralimbic portions, comprises the anterior portion of the frontal lobes. Among other functions, the PFC is typically and most consistently associated with executive function, an umbrella term for higher-order processes such as planning and forethought, problem solving, and cognitive flexibility. With respect to addiction, the PFC regulates the overall motivational significance and determines the intensity of behavioral responding (Goldstein and Volkow, 2011). The output of the PFC is glutamatergic, and is modulated by dopamine and other neuromodulators (Van Eden and Buijs, 2000). Consistent with involvement of dopaminergic afferents, the activation of the PFC by rewarding stimuli is strongly influenced by the predictability of the reward. A hypoactive PFC has been associated with loss of impulse control (Chen et al., 2013), and deficits in PFC function have been observed in patients with a variety of psychiatric illnesses including substance abuse (Goldstein and Volkow, 2011)

and obesity (Cornier et al., 2013), ADHD (Arnsten, 2009), schizophrenia (Yoon et al., 2013), and depression (Koenigs et al., 2008).

The hippocampus is a limbic structure that plays a major role in learning and memory. The hippocampus sends glutamatergic projections to multiple regions within the reward circuitry. In fact, the NAc is innervated by the vHipp subiculum, which is thought to convey contextual and memory-related information. Consistently, direct stimulation of vHipp axons in the NAc reinforces addiction-

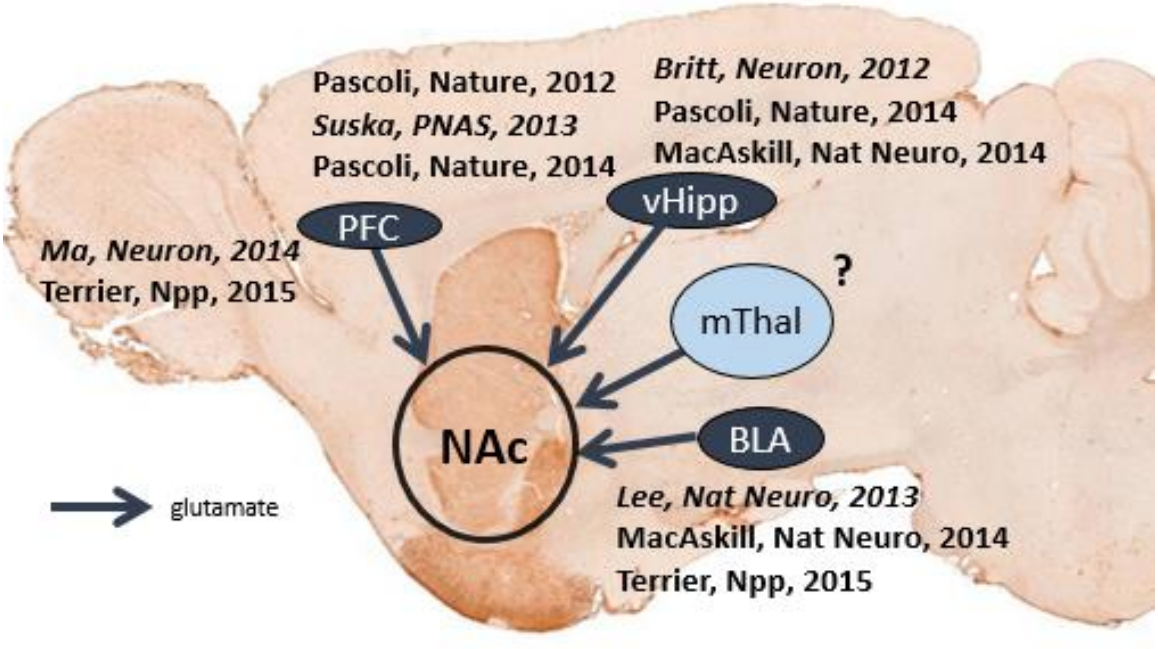


Figure 5. Glutamatergic brain regions projecting to the NAc. Several glutamatergic cortical and limbic regions project to the NAc, which serves to integrate potentially distinct components of motivational information towards the execution of complex tasks. Over the past 4 years, the PFC, vHipp, and BLA inputs to the NAc have been demonstrated to undergo pathological adaptations following cocaine exposure. Italics indicate experiments performed in the rat while the other citations denote mouse experiments with a D1/D2 transgenic reporter as used in this dissertation. Until this body of work, no published account has examined the mThal-NAc circuit in the context of cocaine exposure. The background image was taken from the gensat gene atlas and indicates expression of the A2A subtype adenosine receptor. BLA, basolateral amygdala; mThal, midline nuclei of the thalamus; Nat Neuro, *Nature Neuroscience*; NAc, nucleus accumbens; Npp, *Neuropsychopharmacology*; PFC, prefrontal cortex; PNAS, *Proceedings of the National Academy of Sciences*; vHipp, ventral subiculum of the hippocampus.

related behaviors (Britt et al., 2012) and potentiation of the circuit has been shown to be necessary for reinstatement to cocaine-seeking (Pascoli et al., 2014).

The BLA is a limbic region thought to be necessary for attributing emotional value to cues, thus having an integral role in processing affective (emotional) states (Phelps and LeDoux, 2005). In terms of addiction, the amygdala is thought to be important for cue- and stress-induced reinstatement of drug-seeking behavior. However, lesion studies suggest the BLA is not critical for cocaine self-administration. Similar modulations of amygdala output occur during extinction of fear response and drug-seeking (Peters et al., 2009) and the BLA, along with the extended amygdala, is a likely neurobiological substrate underlying the comorbidity of addiction and anxiety disorders. The BLA therefore acts to integrate the positive or negative value of an environmental stimulus (natural reward, drug of abuse, stress).

The thalamus has been historically conceptualized as a sensorimotor-cortical relay, and has received relatively little attention with regards to its capacity to modulate complex affective and cognitive behaviors (Vertes et al., 2015). Several groups of thalamic nuclei all send and receive information to and from other components of the limbic system, but the mThal are most well known for their ability to regulate reward-related behaviors. Of the mThal nuclei, the paraventricular nucleus (PV) and paratenial nucleus (PT) project most densely to the NAc (Berendse and Groenewegen, 1990). The PVT and PT receive information from a diverse host of reward-related structures (Li and Kirouac, 2012; Phillipson, 1988) including the PFC, vHipp, hypothalamus, bed nucleus of the stria

terminalis (BNST), and the VTA and other midbrain regions. Thus the mThal nuclei are anatomically positioned to provide essential coordination of learned drug-induced behaviors. Indeed, lesion or inhibition of the PVT has been demonstrated to disrupt behaviors conditioned by drugs of abuse (Browning et al., 2014; Hamlin et al., 2008; James et al., 2010; Young and Deutch, 1998), including reinstatement of cocaine-seeking and expression of cocaine CPP.

Although excitatory drive on the NAc-VTA axis is the key final common pathway to addiction, other regions, such as the extended amygdala play a key role in distinct addiction-related behaviors (Koob et al., 1998). The extended amygdala is composed of several basal forebrain regions including the NAc shell, the BNST, and the central nucleus of the amygdala (CeA), all of which have similar morphology, immunoreactivity and connectivity. The extended amygdala is the aforementioned area implicated as a key mediator of stress-induced relapse (S Erb et al., 2001).

Other structures tertiary to the reward circuitry play a role in mediating addiction. For instance, the mesolimbic and nigrostriatal pathways are innervated by a wide variety of brain regions, whose inputs supply information concerning the environment and the animal's motivational and emotional states. One particular region, the hypothalamus, is a highly diverse brain region perhaps best known for its close association with the pituitary gland and the endocrine system. The lateral hypothalamus (LH) which is reciprocally connected to the NAc shell is a target for self-stimulation (Margules and Olds, 1962). Consistent with the critical role of the

LH in metabolic homeostasis and reward, drug valence can be modified by metabolic states (for review see Stice et al., 2012).

2. Rodent Models of Addiction-like Behaviors

Much of our knowledge of addiction and substance abuse comes from preclinical experiments, the vast majority of which are performed in mice or rats. The utility of rodent addiction models are exemplified as follows: there are many variations of rats and mice with strain-specific traits that are particularly useful for modeling aspects of addiction-related behaviors (e.g. high anxiety, alcohol-preferring) and rodents also engage in complex spontaneous and conditioned behaviors that are implicated in substance abuse. Furthermore, mice are highly amenable to genetic manipulation allowing for cell type and region specific manipulation of protein expression. The use of mice or rats also confers pragmatic advantages in that they are small and inexpensive, easy to house and maintain, and reproduce readily and rapidly.

2.1 Neurochemistry

Several quantitative neurochemical techniques are commonly employed to study the neurophysiological effects of abused substances. Microdialysis, in conjunction with liquid chromatography/mass spectrometry, allows for quantification of dopamine, other biogenic amines, and/or glutamate/GABA induced by a substance of abuse. Neurotransmitter collection often occurs while the animal is simultaneously performing passive or contingent addiction-related

behaviors. Additionally, biosensor technology is very similar to microdialysis, except the detection method is enzymatic and allows for more precise measurements over shorter intervals. Finally imaging techniques, including positron emission tomography and magnetic resonance imaging, are used in rodents, primates, and humans to examine how abused substances occupy receptors, induce neurotransmitter release, or alter brain region activation or connectivity.

2.2 Behavioral Models

Unlike most illnesses and some other psychiatric disorders, drug addiction is largely defined by its behavioral components. Effectively reproducing these behaviors in animals is essential to making clinically relevant scientific discoveries. For a complex psychiatric disorder, a comprehensive animal model is likely unattainable. Instead, animal models are designed as a means to examine one or more particular components of a human disorder. When discussing or creating an animal model, there are several types of validity to consider. The first concept, and arguably the most relevant one, is construct validity, which refers to how meaningfully, interpretably, and powerfully the conclusions drawn from the model can apply to the psychiatric condition (Edwards and Koob, 2012). Construct validity commonly refers to the similarity between the underlying biology of the animals and the patients. Alternatively, construct validity relates to the concept of functional equivalence, that a change in one variable (e.g. stress) should similarly affect the outcome (e.g. drug-seeking) in the model and the clinical population (Katz and

Higgins, 2003). For instance, reinstatement to cocaine-seeking in the rat is considered to have a high degree of construct validity relative to relapse since stimuli (e.g. stress, cocaine cues, and cocaine itself) that induce reinstatement are also known to trigger relapse. Additionally, similar biological underpinnings have been identified in humans and rodent models (e.g. deficits in PFC function).

The concept of functional equivalence is closely related to predictive validity, which more specifically refers to the ability to predict the clinical response to an intervention based on the response in the animal model. One should also consider face validity, which refers to how well the animal model resembles components of the psychiatric disorder. Lastly some have proposed considering population validity, an extension of face validity, which dictates that the rate of occurrence of a disease-related behavior should reflect epidemiological data (Schmidt, 2011). For example, only approximately 20% of cocaine users transition to clinical cocaine dependence (Lopez-Quintero et al., 2011), so a model where only 20% of animals engaged in addiction-like behavior (see (Deroche-Gamonet et al., 2004)) would be said to have a high degree of population validity. Arguably, this concept is important to ensure that pathological disease-related behavior is being modeled, as opposed to behavior within the normal adaptive range (Steimer, 2011).

Abused substances can be delivered in two ways, contingently (by the subject) or passively (by the experimenter). The distinction is important for two reasons: (1) pathological substance-seeking is a key component of addiction, whereas the prescribed use of medication does not constitute addiction and (2)

studies have demonstrated that experimenter-delivered and self-administered abused substances can induce profoundly different neurobiological and behavioral effects (for examples, see Markou et al., 1999; Wolf and Ferrario, 2010). However, not all effects of substances of abuse are dependent upon contingent administration thus both contingent and passive treatment regimens have been utilized to study each phase of addiction pathology.

2.3 Etiology of Addiction-like Behavior

Clinical substance dependence is diagnosed when at least 3-of-7 criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria have been met within one year. These criteria include tolerance, withdrawal, escalation, persistence, excessive motivation to obtain the substance, giving up other activities, and perseverance despite self-harm. Considering these criteria is important when discussing an animal model of addiction since, as mentioned, behavioral animal models are designed to mimic only a particular facet of the disorder. Animal models of addiction typically replicate aspects of fewer than three of the DSM criteria, and typically measure only one property of drug action.

2.3.1 Stages of Addiction

The binge/intoxication phase of addiction is studied by examining the initial effects of substance administration to naïve animals. Acute and short subchronic (< 7 days) treatments with abused substances have been shown to transiently or semi-permanently perturb normal physiology and behavior. Additionally, in

contingent administration paradigms, animals tend to escalate their substance intake similarly to humans at the onset of the disorder. Negative affect/withdrawal is modeled by continuing the administration of vehicle in lieu of the abused substance (i.e. extinction) or, in some cases, by completely removing access. Like in patients, drug-seeking behavior in animals often intensifies during the first drug-free session – this phenomenon is referred to as the “extinction burst”. The dwindling of drug-seeking during the extinction phase is not due to a loss, or forgetting, of previous drug-related memories, but instead is an active learning process (McNally, 2013). Acceleration or enhancement of this extinction learning is considered to be a potential avenue for improving clinical outcomes. Reinstatement is a phase of administration in animal models that occurs after an extinction phase. In animals that have undergone extinction (i.e. those that no longer engage in drug-seeking), drug-seeking behavior can be elicited by several manipulations relevant to the preoccupation/relapse phase of addiction. These reinstatement treatments include: a stressor, a small “priming” drug dose, and a cue or context previously paired with substance delivery. All three methods are commonly used in efforts to better understand the etiology of relapse.

2.3.2 Reinforcement

Reinforcement is the ability of a stimulus to modify a measureable dimension of instrumental behavior, typically rate, duration, magnitude, or latency. Positive reinforcement occurs when a behavior results in the presentation of an absent stimulus (e.g. receiving a foot massage), whereas negative reinforcement

occurs when a behavior results in the removal of a present stimulus (e.g. scratching an itch). Positive and negative reinforcement are not mutually exclusive properties; this is perhaps best evidenced by the development of addiction where behavior is initially driven by intoxication and is eventually driven by alleviation of withdrawal. The most flexible and robust model of a substance's reinforcing properties is self-administration.

In the self-administration paradigm, animals are trained in a specific environment such that an instrumental response (e.g. lever-press or hole-poke) results in substance delivery. Food, drink, or alcohol are typically delivered by a hopper or retractable lever, whereas for drugs of abuse, animals are implanted with an intravenous catheter used for systemic drug infusion. A variety of manipulations of the required behavior can be made to gain insight into specific aspects of addiction. For example a progressive ratio schedule has been used to examine motivation, addition of coincidental punishment can be used to study persistence, responding in the absence of drug is used to measure compulsive drug-seeking, and the reinstatement of extinguished responding is a model of relapse (Katz and Higgins, 2003). Additionally some researchers have correlated specific behavioral traits (e.g. persistent or compulsive responding) with changes in receptor expression (Kasanetz et al., 2012), neuron excitability (Chen et al., 2013), and synaptic plasticity (Kasanetz et al., 2010). Patterns of intake during acquisition of cocaine self-administration are also strong predictors of developed behaviors (Belin et al., 2009). The versatility of the paradigm, in tandem with its

validity, perpetuates the view that self-administration is the “gold standard” of behavioral addiction research.

2.3.3 Reward

Models of reward specifically capture information related to the hedonic valence of the drug or stimulus, whether positive or negative. That is, these models can readily detect aversive properties of a stimulus since reward and aversion exist on one spectrum. The most substantial distinction between models of reward and reinforcement is that models of reward typically involve the passive, experimenter-delivered administration of a stimulus and therefore do not address motivation. Intracranial self-stimulation (ICSS) can be used to study reward by pretreating an animal with a drug prior to a training session: drugs of abuse act synergistically with deep-brain electrical stimulation to facilitate instrumental behavior in the ICSS paradigm (Wise, 1996). It is worth noting that, through the advent of optogenetics, stimulation of distinct, specific neuronal pathways has been demonstrated to be sufficient to reinforce (Britt et al., 2012; Kravitz et al., 2012; Lammel et al., 2012; Lobo et al., 2010; Stuber et al., 2011) or punish (Kravitz et al., 2012; Lammel et al., 2012; Stamatakis and Stuber, 2012a) instrumental behavior and such preparations are an ongoing area of research.

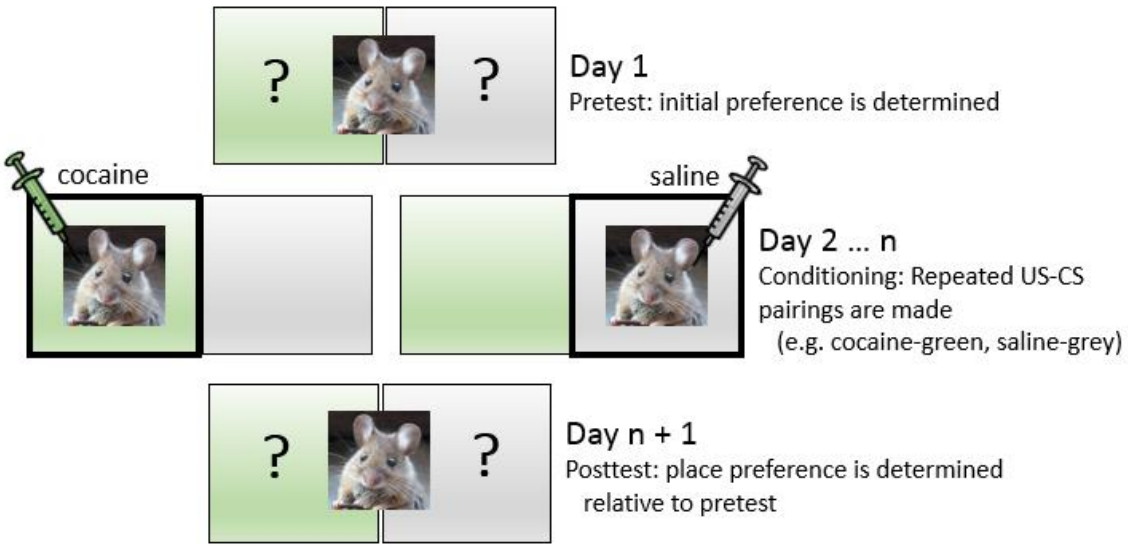


Figure 6. Assessment of reward learning with place conditioning. Mice, other rodents, and primates, can learn to associate the rewarding (or aversive) properties of a stimuli with a particular environment. The assay takes advantage of classical Pavlovian conditioning whereby the unconditioned stimulus (US) whose properties are to be tested (e.g. cocaine) is paired with an otherwise innocuous conditioned stimulus (CS) (e.g. green room). For a place conditioning association to be formed, the stimuli must have inherent emotional valence and not disrupt learning and memory.

Perhaps the most commonly used paradigm to study reward is place conditioning, often referred to as conditioned place preference (CPP) (for reviews, see Tzschentke, 2007, 1998). CPP takes advantage of Pavlovian conditioning in an apparatus with at least two contextually distinct compartments (Figure 6). Animals are conditioned during several training sessions such that a paired association is formed between the stimulus of interest (unconditioned stimulus, US) and a particular chamber in the apparatus (conditioned stimulus, CS). After the completion of the training sessions, the animal is given unrestricted access to all chambers of the apparatus and the preference or avoidance of the conditioned side is taken as a measure of reward or aversion. CPP has been used to study

not just drugs of abuse, but also the rewarding properties of palatable food and drink, novel objects, voluntary exercise, social interaction, copulation, and direct neuronal stimulation. On the other hand, place conditioning can been used to study the aversive properties of acute drug treatment, drug withdrawal, and painful stimuli. Like the self-administration paradigm, CPP can be used to study abstinence and relapse by observing extinction learning and reinstatement induced by cues, stress, or drug priming. One important caveat to CPP is that the expression of a place preference necessitates the learned formation of a paired US-CS association, and the effects of amnestic drugs must be interpreted carefully (Bardo and Bevins, 2000).

Certain anticipatory behaviors have also been used as a measure of the rewarding properties of drugs and natural rewards. Measuring these behaviors requires conditioning an animal such that the conditions of drug delivery or food presentation are held constant and occur at the same manner each time. Like Pavlov's iconic salivating dogs, rodents will engage in specific behaviors in the time period immediately before and after reward delivery. These behaviors, including such quantifiable events as digging and rearing (Labouèbe et al., 2013) and bouts of high-frequency ultrasonic vocalizations (USVs) (Browning et al., 2011; Wright et al., 2012), are often measured during performance of another task, especially self-administration or CPP. Analogous behaviors can also be used to study the aversive properties of drugs (Burgdorf et al., 2001) or drug withdrawal (Covington and Miczek, 2003).

Novelty-seeking and sensation-seeking are personality traits that have been associated with a propensity to use drugs of abuse. There are a few reports of modeling these traits in rodents. For example, rats that strongly prefer environmental novelty are more likely to develop addiction-like cocaine self-administration (Belin et al., 2011). A newly developed rodent model is Operant Sensation Seeking (OSS), in which an animal actively responds for the presentation of dynamic sensory stimuli. OSS performance appears to be dependent on similar molecular substrates as psychostimulant self-administration (Olsen and Winder, 2009; Olsen et al., 2010).

2.3.4 Interoceptive State and Drug Action

The drug discrimination assay is used to model the subjective effects of a drug, also referred to as the interoceptive state. In a human drug user or test subject, the interoceptive state does not necessarily carry emotional valence (as in feeling ‘good’, ‘bad’, or ‘high’), as drugs that are neither rewarding nor aversive can still be detected. There are many different ways to implement a drug discrimination experiment, but two-choice operant paradigms are most commonly used. An animal is trained in a two-choice box where one choice (e.g. left lever press) results in food delivery on days when the animal has been administered drug, while the other choice (e.g. right lever press) is reinforced when the animal has been treated with vehicle. This paradigm is very sensitive and relatively specific for particular pharmacological mechanisms. Substitution studies and antagonism studies have been used to gain insight into the abuse liability and

mechanism of action of psychoactive drugs. Additionally it should be noted that a drug need not have rewarding or locomotive properties to be used as a training or probe drug in drug discrimination; animals have been successfully trained on rewarding (cocaine, morphine), aversive (atropine, naloxone), and neutral (buspirone, clozapine, desipramine) agents (Young, 2009).

Changes in locomotor activity (i.e. either hyperactivity or sedation) are a commonly used measure of drug action as the NAc plays a role in regulating locomotor output. These data must be interpreted carefully since high doses of particular drugs, especially psychostimulants, produce repetitive stereotypical behaviors that interfere with locomotion. In some cases these stereotypies can be very informative, as some have been linked to a specific molecular mechanism of action (e.g. yawning/D3 receptor, head-bobbing/5-HT_{2A} receptor). One important applied paradigm of locomotor activity is sensitization. Like in human addicts (Sax and Strakowski, 2001), rodents will become more sensitive to particular properties of certain drugs during the initial exposure period. For example locomotion and grooming behavior will continue to increase over the first several injects in a chronic stimulant period. This is believed to be related to the dose escalation that is observed in the initial stages of addiction.

2.3.5 Cognition

A wide range of deficits in cognition have been observed in addicted patients. Although there are several potential explanations, current evidence suggests that drug abuse may cause certain cognitive deficits, and conversely

certain cognitive deficits may cause a predisposition to abuse drugs and/or become addicted to them. The brain structure most commonly associated with these particular cognitive deficits is the PFC, and altered cortical function has been observed in both human drug abusers as well as chronically-treated animals. The major cognitive deficits generally associated with addiction are related to attention and problem solving. Although most clinical studies have been correlational, it is generally thought that various cognitive traits confer vulnerability to addiction and that substance abuse itself can alter certain aspects of cognition.

One such aspect is impulsivity, a personality trait that is characterized by the tendency to make quick, rash decisions as well as an inability to cease inappropriate behavior. Impulsivity can be broadly split into two major components: quick and/or rash decision-making (impulsive choice) and disinhibition of motor responses (impulsive action) (Jupp et al., 2013). Impulsive choice is modeled in animals primarily via delay discounting, a paradigm that measures the preference for small, immediate rewards vs. large, delayed rewards. On the other hand, impulsive action is modeled in several paradigms, one of which is the Go/No-Go task, where the animal must cease responding when a tone is presented during interspersed “no-go” trials (Rodriguez and Wetsel, 2006).

Another cognitive domain believed to be involved in addiction is executive function. Executive function refers to a group of “higher order” tasks including problem solving and cognitive flexibility, and is thought to be mediated in large part by the PFC. One commonly-used clinical assay to detect deficits in executive function is the Wisconsin Cord Sort Task, while such deficits are assessed in

animals in reversal learning paradigms and in the attentional set-shift task (Dias et al., 1996). PFC-mediated “top-down” control of subcortical structures is thought to suppress drug-seeking behavior following extinction training (Peters et al., 2009).

2.3.6 Anxiety and Anhedonia

Like cognition, a complex bidirectional relationship exists between addiction and stress/anxiety. At disease onset, drugs can be sought as a means of relieving stress. Eventually drug withdrawal becomes a stressful experience, and, in rehabilitated individuals, stress can trigger relapse to drug seeking behavior. While many anxiety models exist, the canonical model of anxiety in rodents is the elevated plus maze, an arena that consists of one open, vulnerable arm and one closed, sheltered arm. When placed in an elevated maze, rodents exhibit an approach-avoidance conflict between attraction to novel environments and aversion to heightened and/or open spaces. The proportion of time spent in the closed arms, taken as a measure of anxiety, is reliably increased and decreased by anxiogenic and anxiolytic manipulations, respectively (Rodriguez and Wetsel, 2006).

Major Depressive Disorder, and its anhedonic symptoms in particular, is often comorbid with substance use disorders. Anhedonia is an inability to enjoy pleasurable activities, and often occurs during acute or protracted withdrawal from drugs of abuse. Some of the addiction-related animal models, like ICSS and CPP, have even been used to model anhedonia (Lim et al., 2012). Additionally, sucrose preference is often used to study anhedonia in rodents. Animals are given equal

access to standard drinking water and an otherwise identical solution sweetened with sucrose or saccharin. Stressful conditions and drug withdrawal can decrease the preference for sucrose, and chronic antidepressant treatment can reverse that effect (Der-Avakian and Markou, 2010; Strekalova et al., 2011).

3. Molecular Mediators of Addiction Pathology

Addiction is conceptualized as a learning disorder, whereby drugs of abuse hijack the same glutamate-dependent cellular mechanisms that enable learning and memory. Synaptic plasticity is a collective term for when patterns of neural activity alter the strength of the connection between two neurons. Synaptic plasticity occurs at both excitatory and inhibitory synapses and may be mediated by altering the number of synapses, quantal size, probability of neurotransmitter release, or the functional/expression of neurotransmitter receptors. Long term changes in synaptic strength and connectivity are also mediated by structural changes in form and function, including changes in the expression of receptors and channels, the localization of cytoskeletal and scaffolding proteins, and the activation state of kinases, phosphatases, and transcription factors (Russo et al., 2010).

3.1 Structural and Regulatory Proteins

Structural proteins are critical for the development, maintenance and plasticity of excitatory synapses. As exposure to drugs of abuse leads to remodeling of excitatory synapses, it is not surprising that synaptic scaffolding

proteins are important proteins implicated in drug-related behaviors. To date, postsynaptic structural proteins including (but not limited to) postsynaptic density 95 (Yao et al., 2004), Kalirin (Wang et al., 2013), activity-regulated cytoskeleton-associated protein (A. L. Brown et al., 2011), A-kinase anchor protein (Reissner et al., 2011), integrins (Wiggins et al., 2011), spinophilin and neurabin (Allen et al., 2006), and Homers (Szumlinski et al., 2006) have been implicated in drug related behaviors. For the most part, with the exception of Homers discussed below, these structural proteins act to form and maintain synapses and also stabilize the expression and function of glutamate receptors.

One of the most thoroughly studied scaffolding proteins in regards to addiction-related phenomena are the Homer proteins. Homers are scaffolding proteins that regulate cell signaling by regulating Group 1 metabotropic glutamate receptor (mGluR) trafficking and extracellular glutamate concentrations (Szumlinski et al., 2008). Homers are also involved in dendritic spine enlargement and postsynaptic density maturation. Evidence has pointed towards Homer proteins as being crucial for the long-lasting synaptic and behavioral plasticity following drug administration. For example, genetic deletion and viral-mediated rescue demonstrated that Homer2 is essential for the neuroplastic effects and rewarding properties of alcohol (Szumlinski et al., 2005). By contrast, deletion of either Homer1 or Homer2, has been shown to sensitize mice to neurochemical and behavioral changes induced by cocaine (Szumlinski et al., 2004).

Many drugs of abuse directly bind or functionally interact with the ER-associated intracellular chaperone known as the σ1 receptor (Maurice and Su,

2009). The σ1 receptor is thought to co-localize with inositol triphosphate receptors and modulate intracellular calcium release. σ1 activity also regulates transporters and ligand- and voltage-gated ion channels and has been implicated in the reinforcing and addictive properties of psychostimulants (Katz et al., 2011).

3.2 Kinases

One of the most pronounced signaling pathways in addiction includes elements of the adenylate cyclase, cAMP, and protein kinase A (PKA) signaling pathway. PKA is thought to be activated by D1R signaling in response to drug exposure. Among other events, this leads to phosphorylation of AMPARs and convergence with the protein kinase C pathway onto the extracellular-related kinase (ERK) pathway. Activation of this pathway has been reported following acute and repeated treatment with cocaine, amphetamine, THC, nicotine, and morphine in the VTA, NAc, extended amygdala, and PFC (Zhai et al., 2008). Additionally, the ERK pathway is suggested to be dependent on the phosphatase inhibitor dopamine- and cAMP-regulated phosphoprotein-32 (Gerfen et al., 2008; Zhang et al., 2006). Cyclin-dependent kinase 5 (Cdk5), another key component in the pathway, is also implicated in addiction-related behaviors since chronic cocaine exposure has been shown to increase Cdk5 levels in the striatum (Bibb et al., 2001). This pathway plays a variety of diverse roles in different brain regions and disease stages. In the NAc for example, the ERK pathway mediates some of the initial effects of cocaine (Pascoli et al., 2012), while in the central amygdala it plays a role in the incubation of cocaine craving (Lu et al., 2005). ERK activation

has been shown to interact with the epigenetic machinery, including the expression of immediate early genes and transcription factors.

3.3 Transcription Factors

A variety of epigenetic mechanisms are thought to contribute to the persistent plastic changes related to addiction. Histone modification, DNA methylation, and the production of non-coding RNA and transcription factors have all been implicated in the pathophysiology of substance abuse (Robison and Nestler, 2011). The two transcription factors most extensively studied with respect to addiction are ΔFosB (Chen et al., 1995; Grueter et al., 2013; Hiroi et al., 1998, 1997; Lobo et al., 2013) and cAMP response element binding protein (CREB) (T. E. Brown et al., 2011; Dong et al., 2006; Huang et al., 2008; Konradi et al., 1996, 1994). These transcription factors are upregulated following exposure to drugs of abuse and likely lead to upregulation of mRNA of Cdk5 (Bibb et al., 2001). Overexpression of either ΔFosB (Kelz et al., 1999) or CREB (Larson et al., 2011) within the striatum has been shown to potentiate the behavioral effects of cocaine, likely through similar mechanisms of gene expression regulation (McClung and Nestler, 2003). Interestingly, a recent study has demonstrated that selective overexpression of ΔFosB within D1R-, but not D2R-, expressing neurons in the NAc potentiates behavioral responses to cocaine (Grueter et al., 2013). Others have shown that ΔFosB is necessary and sufficient for the cocaine-mediated synaptic remodeling and CaMKII induction in D1-expressing neurons (Robison et

al., 2013). Taken together these data highlight the need for the continued need for cell-type specific experimental designs in future research efforts.

3.4 Cell Surface Receptors and Transporters

In addition to signaling through ligand-gated ion channels, glutamate also sends modulatory signals through mGluRs. While activation of Group II mGluRs has been shown to attenuate reinstatement of seeking of cocaine (Baptista et al., 2004) and heroin (Bossert et al., 2005), the G_q-coupled Group 1 mGluRs have been the most thoroughly studied with respect to drug abuse, particularly $mGlu_5$ (Grueter BA et al., 2008). Experiments utilizing selective negative allosteric modulators or knockout mice have implicated $mGlu_5$ in the rewarding, reinforcing, and motivational properties of several classes of abused substances (Amato et al., 2013; Bird and Lawrence, 2009; Grueter et al., 2007) and also dynamic sensory stimuli (Olsen et al., 2010). Furthermore, acute cocaine exposure has been shown to lead to intracellular sequestration of $mGlu_5$ via a Homer-dependent manner (Szumlinski et al., 2006), while chronic cocaine administration has been shown to upregulate $mGlu_5$ in both the NAc (Ghasemzadeh et al., 1999) and hippocampus (Freeman et al., 2001). In some brain regions, activation of Group 1 mGluRs has also been linked to the production of endocannabinoids (eCBs).

In addition to playing a well-known role in stress and anxiety (Ramikie and Patel, 2012), the eCB system has been implicated in addiction-related processes and behaviors (Tanda, 2007). In fact, drugs of abuse and natural rewards are known to alter brain eCB content (Solinas et al., 2007). Endocannabinoids

attenuate neurotransmitter release through at least two targets, the $G_{i/o}$-coupled cannabinoid 1 receptor (CB1R) and the transient receptor potential vanilloid 1 (TRPV1). In addition to being activated by tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana, antagonism or genetic deletion of CB1R has been shown to suppress reinstatement of several class of drugs (Serrano and Parsons, 2011). Additionally, CB1R activity is required for reinstatement of sucrose (Vries et al., 2005) and corn oil (Ward et al., 2007) self-administration, suggesting that CB1R mediates a non-selective motivational component of conditioned reinforcement. Much less is known about TRPV1 and reward processes, but studies utilizing TRPV1 knockout mice suggest that the channel plays a role in the behavioral effects of ethanol (Blednov and Harris, 2009) and cocaine (Grueter et al., 2010).

In clinical populations few targets have been studied as much as the plasma membrane dopamine transporter (DAT) and dopamine receptors. PET studies have revealed decreased availability of DAT and D2-like receptors in patients who abuse several classes of drugs (Volkow et al., 2009) as well as in patients with obesity (Wang et al., 2001). It should be noted that the majority of these studies were performed using a radiotracer ([^{11}C]-raclopride) with high affinity for both D2 and D3 subtype receptors. D3R is structurally and functionally homologous to D2R, but is more strictly localized to the mesolimbic circuit, specifically the NAc shell and the islands of Calleja (Gangarossa et al., 2013). In contrast to the reports using labeled raclopride, one recent PET study that used a D3R-preferring ligand observed upregulation of D3Rs in methamphetamine abusers (Boileau et al.,

2012). Additionally, evidence from several preclinical models implicate D3R activation in the behavioral effects of psychostimulants (Newman et al., 2012). In rodent models, dopamine receptor antagonists decrease the reinforcing effects of cocaine when administered peripherally (Caine and Koob, 1994; Everitt and Wolf, 2002). Restricted drug infusions have elaborated that the NAc is at least one essential region for D1-like and D2-like antagonists to attenuate cocaine reinforcement (Bari and Pierce, 2005; Caine et al., 1995). Finally, in addition to affecting dopamine receptors and its plasma membrane transporter, psychostimulant abuse may perturb the packaging of dopamine into synaptic vesicles. Studies have linked both cocaine (Narendran et al., 2012) and methamphetamine (Johanson et al., 2006) abuse with decreased vesicular monoamine transporter availability in the striatum.

CHAPTER II

SYNAPTIC PLASTICITY AND ADDICTION

1. Ionotropic Glutamate Receptors

The common thread that links the neuromodulatory systems discussed in Chapter I is that their signaling pathways converge on the function of ionotropic glutamate receptors (iGluRs). iGluRs provide the vast majority of fast excitatory neurotransmission in the CNS, and are therefore the predominant targets of processes that govern learning and memory. iGluRs were originally dichotomized based on whether they are activated by the glutamate analog N-methyl-D-aspartate (NMDA). Over the past several decades, major structural and functional differences between NMDA-subtype glutamate receptors (NMDARs) and non-NMDARs have been demonstrated to confer a host of profound differences in their biology (Paoletti and Neyton, 2007). One defining feature is that the channel pore of most NMDARs is blocked by Mg^{2+} ions at resting membrane potentials, so the receptor remains closed even in the presence of endogenous ligands. Depolarization of the cell membrane causes a conformational shift that relieves the magnesium block and confers responsiveness to glutamate. For this reason, NMDARs have been referred to as molecular coincidence detectors that only open in the presence of two agonists as well as coincidental strong neuronal activity.

NMDARs are composed of two obligatory d-serine/glycine-binding GluN1 subunits and two glutamate-binding GluN2A-D subunits (Paoletti et al., 2013). The milieu of synaptic NMDAR subtypes can strongly influence the induction of

synaptic plasticity. While d-serine/glycine presence is required for NMDAR activation and several *Grin1* splicing variants are common, variation in expression of the GluN2 subunit is thought to convey most of the functional diversity observed in native NMDARs. GluN2A and GluN2B are the most abundantly expressed GluN2 subunits in the CNS (Paoletti and Neyton, 2007), and assemble as both homodimers (i.e. GluN1-GluN1-GluN2A-GluN2A and GluN1-GluN1-GluN2B-GluN2B) and heterotrimers (GluN1-GluN1-GluN2A-GluN2B). GluN2B-expressing NMDARs are also enriched at silent synapses (Lee and Dong, 2011), which are essential components of CNS development and have an increasingly-appreciated role in learning and memory in adults. In addition to subunit-specific protein-protein interactions (Robison et al., 2005; Sanz-Clemente et al., 2013), GluN2B-containing NMDARs exhibit prolonged decay kinetics and therefore flux more calcium per channel opening event (Paoletti et al., 2013; Traynelis et al., 2010). Although less abundantly expressed, NMDARs containing GluN2C and GluN2D display even longer decay kinetics than those expressing GluN2B or GluN2A (Vicini et al., 1998). Additionally, GluN2C- and GluN2D-containing NMDARs pass relatively more current at hyperpolarized potentials, and may even open at resting membrane potentials (Schwartz et al., 2012; Seif et al., 2013).

Aside from NMDARs, the major iGluR class involved in excitatory postsynaptic signaling complexes is the AMPA-subtype receptor (AMPAR), glutamate-gated ion channels that flux sodium and other potassium resulting in depolarization of the synaptic membrane. AMPARs are considered to be the "workhorse" of the excitatory synapse responsible for basal transmission. AMPAR-

dependent depolarization of the neuron can lead to action potential firing and thus propagation of the electrochemical signal through the circuit. Sufficient AMPAR-mediated depolarization can relieve the magnesium block of NMDARs; therefore NMDARs open following strong and/or coincidental synaptic activation. Unlike most AMPARs, NMDARs play a key role in synaptic plasticity due to the fact that they are permeable to calcium. Calcium flux through NMDARs results in the activation of calcium-dependent signaling cascades that can lead to transient or long-lasting changes in synaptic strength (Shonesy et al., 2014) (Figure 7). Metabotropic interactions between NMDARs and plasticity-related signaling partners (e.g. Src) have also been identified.

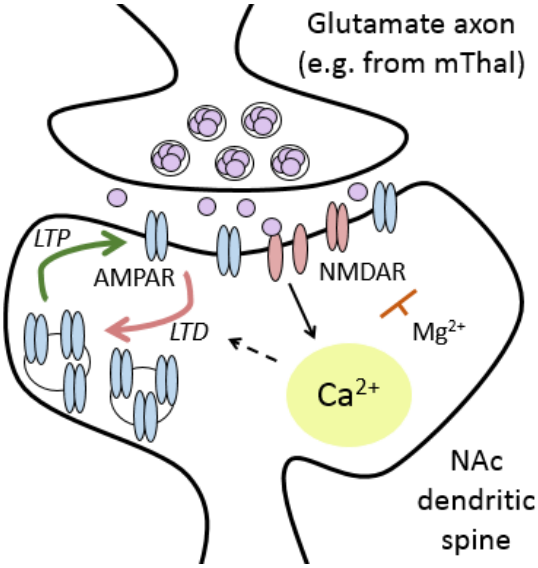


Figure 7. Simplified schematic of excitatory synapse in NAc. Glutamate is released from terminals after which it binds N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). At resting membrane potentials, NMDARs are gated by Mg^{2+}. However, following strong and/or coincidental synaptic activity, depolarization relieves the Mg^{2+} block, permitting NMDAR activation and subsequent Ca^{2+} influx. Elevations of synaptic Ca^{2+} can then precipitate a variety of signaling cascades, some of which alter the function of AMPARs. NMDAR-mediated strengthening and weakening of AMPAR surface expression (i.e, long-term potentiation, LTP, and long-term depression, LTD) are two major molecular mechanisms underlying learning and memory.

Persistent increases or decreases in synaptic strength can be classified as either long-term potentiation (LTP) or long-term depression (LTD), respectively (Malenka and Bear, 2004). A wide variety of studies have demonstrated that

substances of abuse dynamically modulate synaptic transmission within several brain regions, including the VTA and NAc (Lüscher and Malenka, 2011), supporting the concept that synaptic plasticity is a key molecular constituent of experience-dependent behavioral plasticity.

2. Plasticity in the VTA

VTA neurons integrate reward and aversion related information from distinct excitatory inputs (Lammel et al., 2012). Since the first account of drug-induced synaptic plasticity (Ungless et al., 2001), which demonstrated that a single dose of cocaine could precipitate LTP-like changes at VTA dopaminergic neurons, many iterations of the phenomena have been uncovered (Lüscher and Malenka, 2011). Similar results have since been obtained following administration of other drugs of abuse including amphetamine, morphine, nicotine, and ethanol (Saal et al., 2003). Importantly, such changes in VTA synaptic transmission were not observed following similar treatment with non-addictive, psychoactive drugs such as the antidepressant fluoxetine (Saal et al., 2003). Additionally, LTP of GABAergic synapses (i-LTP) has been observed at NAc-VTA synapses following cocaine exposure (Bocklisch et al., 2013).

3. Plasticity in the NAc

3.1 First Accounts

In the NAc changes in excitatory synaptic strength have proven to be more complex. Increasing evidence advocates that dynamic changes in synaptic

function occur in a synapse-specific and experience-dependent manner (Grueter et al., 2012). The foundation for current research lies in seminal studies that suggest synaptic strength at excitatory synapses onto NAc shell MSNs decreases following re-exposure to cocaine (Thomas et al., 2001). This first report did not detect any changes in excitatory synaptic strength in the NAc core. Two primary findings suggested that cocaine sensitization decreased synaptic strength in the NAc shell. For one, the authors reported diminished induction of NMDAR-dependent LTD (3 bouts of 3 min, 5 Hz stimulation). The authors also determined that the amplitude of asynchronous EPSCs (asEPSCs) elicited in strontium-replaced artificial cerebrospinal fluid (aCSF) was decreased following cocaine sensitization. Interestingly, the amplitude of miniature EPSCs (mEPSCs) was unaffected, and the authors concluded that – given the placement of the stimulating electrode – asEPSCs primarily reflected PFC-NAc shell quantal size. Taken together these data suggest that a sensitized cocaine challenge decreases the function of AMPARs at PFC-NAc shell synapses.

Of note, these early experiments were all performed in the context of a cocaine challenge injection (5 x sal + coc challenge vs. 5 x coc + coc challenge). Later research would reveal that the state of cocaine exposure dynamically modulates synaptic strength in the NAc. Kourrich et al. elaborated on the initial findings and revealed that excitatory signaling in the NAc shell is potentiated following extended withdrawal, and then subsequently depressed following drug re-exposure. A single injection of cocaine (while exerting a pronounced effect on $mGlu_5$ function (Fourgeaud et al., 2004; Grueter et al., 2010), was demonstrated

to minimally affect iGluR function in the NAc shell. Other groups have since corroborated these findings via biochemistry (Boudreau and Wolf, 2005; Boudreau et al., 2007; Ghasemzadeh et al., 2003; Robison et al., 2013). A variety of salient stimuli, including stressors, novelty, amphetamine, and other experiences linked to relapse, have also been demonstrated to generate changes in NAc synaptic physiology similar to those induced by cocaine (Jedynak et al., 2015; Rothwell et al., 2011a, 2011b)

Demonstrating a causal relationship between abnormal behavior and altered synaptic plasticity has been a persistent challenge in the field of behavioral neuroscience. In 2005, Brebner et al. became the first to publish a test a causal relationship between AMPAR surface expression and locomotor sensitization. The authors first characterized a putative NMDAR-dependent LTD protocol (8 min, 1Hz, paired with depolarization). By using dominant negative dynamin and GluA2 peptides, the authors characterized this LTD as proceeding through clathrin-dependent AMPAR endocytosis. Then, when delivered systemically or locally into the NAc, the (Tat-fused) GluA2 peptide inhibited the expression of locomotor sensitization to amphetamine. Peptide infusion into the VTA exerted no effect, emphasizing that excitatory synaptic plasticity in the NAc is paramount for long-lasting behavioral effects of psychostimulants. Despite concerns over the GluA2 peptide specificity, these experiments were the first to suggest that NAc LTD is not only correlated with behavioral sensitization, but is in fact a necessary mechanistic component.

Although locomotor sensitization has been proposed to model certain facets of substance use disorders, it is inarguable that drug self-administration more closely recapitulates the human condition. One phenomenon that has been particularly well-studied is the incubation of cocaine craving (Caprioli et al., 2015; Conrad et al., 2008; Lee et al., 2013; Loweth et al., 2014; Lu et al., 2005; Ma et al., 2014). After rodents have been trained to self-administer cocaine, incubation refers to the enhancement of cue-induced drug-seeking observed following extended abstinence. The first link between incubation of cocaine craving and NAc AMPAR function was published by Conrad et al., 2008. Extended withdrawal from cocaine self-administration was associated with an increase in GluA1 and GluA3 expression in the NAc as assessed by Western Blot.

Assembly of AMPARs containing only GluA1 and GluA3 (sometimes called GluA2-lacking AMPARs) is of special interest due to their low basal expression in the MSNs, high channel conductance, and permeability to calcium. For the last reason, they are referred to as calcium-permeable AMPARs (CP-AMPARs). Conrad et al. functionally corroborated that abstinence from cocaine self-administration enhanced function of NAc CP-AMPARs by an increase in AMPAR inward rectification and sensitivity to 1-napthylacetylspermine (Naspm), a selective CP-AMPAR blocker. Furthermore, local infusion of Naspm into the NAc inhibited incubation of cocaine craving. Since this report, others have demonstrated that $mGlu_1$ activation in the NAc shell can internalize CP-AMPARs and similarly blunt cue-induced cocaine-seeking (Loweth et al., 2014). Also, individual differences in addiction-like self-administration have been linked to impairments in PFC-NAc

LTD (Kasanetz et al., 2010) and Layer V pyramidal neuron excitability (Chen et al., 2013).

3.2 Cell Type Specificity

Since the development of BAC transgenic reporter mouse lines, several groups have reported that cocaine-induced changes to NAc excitatory signaling are localized to D1 MSNs (Bock et al., 2013; Dobi et al., 2011; Pascoli et al., 2014, 2012), although changes to D2 MSNs have also been reported (Bock et al., 2013; MacAskill et al., 2014). Dobi et al. found that non-contingent cocaine administration potentiated glutamate synapses specifically on D1-expressing MSNs, and that withdrawal was not required for some of these observed synaptic effects. Of note, the authors measured spine density in parallel to corroborate the electrophysiological findings. Since then, two independent reports have used glutamate uncaging to demonstrated that cocaine exposure modulates AMPAR surface function specifically on D1-MSNs (Bock et al., 2013; Khibnik et al., 2015).

Maintenance of synaptic potentiation following cocaine exposure has been hypothesized to require long-lasting increases in the expression of transcription factors (Russo et al., 2010). The upregulation of many such factors has been reported to occur mainly on D1-MSNs following drug exposure (Lobo and Nestler, 2011). For example, cocaine administration has been demonstrated to increase the phosphorylation of extracellular signal-regulated kinase (p-ERK), mitogen- and stress-activated kinase-1 (p-MSK1), and histone H3 (p-H3) specifically in D1-MSNs (Bertran-Gonzalez et al., 2008). Induction of c-fos and zif268 was also

localized to D1-MSNs. Another transcription factor heavily implicated in NAc-dependent synaptic and behavioral plasticities is ΔFosB (Hiroi et al., 1997; McClung and Nestler, 2003). Cocaine and other salient stimuli are well-known to induce ΔFosB expression in the NAc, and recent work has localized this induction specifically to D1-MSNs (Lobo et al., 2013). Consistently, overexpression of ΔFosB in NAc D1-MSNs recapitulates alterations in synaptic physiology induced by cocaine and enhances the acquisition of cocaine conditioned place preference (Grueter et al., 2013). Despite these advancements in isolating how distinct NAc neuron populations are affected by drug exposure, understanding the contributions of the various NAc glutamate afferents has only just begun.

3.3 Input Specificity

In addition to examining the cell type-specificity of these phenomena, several recent studies have demonstrated that cocaine-induced dysregulation of glutamatergic signaling occurs at specific afferents to the NAc and is necessary for relapse-related behaviors (Britt et al., 2012; Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014). While not targeting specific MSN types, Britt et al. analyzed the synaptic and behavioral profile of the PFC, BLA, and vHipp afferents to the NAc shell. The authors found that vHipp afferents were the densest of the three and accordingly elicited the largest EPSCs in the region. While exhibiting similar AMPAR function at baseline (AMPAR/NMDAR and asEPSC amplitude), vHipp synapses were uniquely potentiated following experimenter-delivered cocaine administration. Furthermore, activation and inhibition of vHipp-NAc terminals using

in vivo optogenetics was demonstrated to enhance and suppress the cocaine-induced hyperlocomotion. Finally, stimulation of each of any glutamate terminal in the NAc was found to be both rewarding and reinforcing by performance on a real-time place preference task and operant self-administration (in contrast to Stuber et al., who did not find the PFC-NAc pathway to be reinforcing).

Since Britt et al. published the first *ex vivo* physiology study to tease apart distinct NAc input functions, a flurry of reports has surfaced. In 2012, Pascoli et al. published that a single injection of cocaine potentiates NAc shell D1-MSN AMPAR function one week later, as assessed by mEPSC amplitude. The authors also reported occlusion of NMDAR- and ERK-dependent LTP – a notoriously difficult process to elicit in the NAc reported to occur only in young mice (Schramm et al., 2002). Nonetheless, the authors set out to depotentiate these synapses through the use of optogenetics. Indeed, they found that PFC-NAc shell synapses exhibited enhanced LFS-LTD (10 min, 1 Hz) following cocaine exposure. Finally, to test the causative relationship between the potentiated state of NAc shell synapses and cocaine-conditioned behaviors, they applied the PFC-NAc LFS *in vivo* and were able to block the expression of locomotor sensitization.

Further work from the Luscher lab aimed to examine excitatory signaling at the specific NAc shell inputs in the context of reinstatement to drug seeking. The authors found that D1-MSNs were selectively altered following cocaine self-administration at inputs from the PFC and vHipp, but not BLA. PFC-D1(+) NAc shell synapses were characterized by enhanced expression of CP-AMPARs, while vHipp-D1(+) by increased EPSC size and AMPAR/NMDAR. The authors then

characterized 1 Hz and 13 Hz plasticity at each input, and found the protocols induced 4 qualitatively different of homosynaptic and heterosynaptic plasticities When applied *in vivo*, each stimulation differentially affected aspects of cue-induced cocaine-seeking behavior, with 13 Hz PFC stimulation abolishing all drug seeking. Of note, PFC-NAc shell LFS (10 min, 1 Hz) – as featured in the 2012 report – induced less LTD following cocaine exposure and did not affect drug-seeking, supposedly emphasizing differences between non-contingent and contingent drug-administration paradigms.

Despite these potential differences, a recent report out of Pascoli's lab (Terrier et al., 2015) confirmed that PFC-D1(+) NAc shell synapses display CP-AMPARs following self-administered cocaine, and extended those findings to a single experimenter-delivered injection of cocaine. While these findings corroborate one report from the Luscher lab (Mameli et al., 2009), they remain at odds with those from other groups (Kourrich et al., 2007; McCutcheon et al., 2011). Like the labs of Mark Thomas and Marina Wolf, I did not observe altered AMPAR rectification on NAc shell D1-MSNs following non-contingent cocaine exposure, after searching with PFC-specific, mThal-specific, and electrical stimulation (unpublished results, data not shown). Nonetheless, in addition to changes in CP-AMPAR assembly on D1-MSNs following any form of cocaine exposure, the authors noted that self-administration of an extremely high dose of cocaine (1.5 mg/kg/infusion) enhanced CP-AMPAR function on D2-MSNs. They continued by identifying the relevant source of glutamate as the BLA. The authors attributed this phenomenon as resultant from the aversive properties of such a high dose.

Consistently, AMPAR potentiation at PVT-D2(+) NAc shell synapses following morphine withdrawal has recently been reported (Zhu et al., 2016).

Adam Carter and his lab have taken a unique biophysical approach to understanding neurocircuit functions. In an initial account (MacAskill et al., 2012), his group discovered that while all NAc MSNs receive afferents from each region examined (PFC, mThal, vHipp), vHipp connections onto D2-MSNs were uniquely weak, as evidenced by minimal spike probability and diminished EPSC amplitudes relative to paired D1-MSNs. The weak connection of the vHipp-D2(+) circuit was then attributed to unique subcellular connections, in that vHipp inputs were connected to smaller spines located further away from the soma. While these experiments provide intriguing physical context for basal differences in excitatory NAc circuit function, the relevance for drug-related memory formation and behavior was not addressed.

In a follow-up study (MacAskill et al., 2014), the group examined how vHipp- and BLA-NAc shell circuit function is altered during short-term abstinence (3 days) from 5 days of cocaine exposure. The paradigm increased mEPSC frequency and spine density on D1 MSNs, suggesting a reorganization of excitatory connectivity during this transformative period. The authors then demonstrated that the cocaine-induced increase in spine density occurred specifically at BLA-D1(+) synapses, whereas vHipp-D1(+) synapses displayed decreases in spine volume. Finally, chemogenetic inhibition of the BLA during cocaine administration inhibited the BLA-NAc reorganization and the expression of behavioral sensitization, but did not affect changes to vHipp-NAc synaptic architecture. In contrast, inhibition of the

vHipp during cocaine administration maintained vHipp-NAc connectivity, but did not prevent BLA-NAc restructuring or the expression of locomotor sensitization. Taken together these experiments revealed several fascinating physical processes that ultimately underlie drug-induced learning and memory.

As a whole the body of work illustrates that long-term physiological and behavioral changes induced by cocaine exposure require LTP-like changes in the NAc, consistently including the functional upregulation of AMPARs. In fact, rapid LTD-like internalization of AMPARs may be required the for the retrieval of drug-conditioned memory (Brebner et al., 2005). Many groups, but not all, have reported that cocaine experiences increase the expression of calcium-permeable, GluA2-lacking AMPARs in the NAc, especially (and specifically) following self-administered cocaine (Boudreau and Wolf, 2005; Conrad et al., 2008; Lee et al., 2013; Ma et al., 2014; McCutcheon et al., 2011; Pascoli et al., 2014).

4. NMDAR Function

Most of the changes to excitatory synaptic strength or plasticity that occur have been proposed to require NMDAR activity during the cocaine experience (Cahill et al., 2014; MacAskill et al., 2014; Pascoli et al., 2012). However the data describing how cocaine may alter NMDAR function is less clear. Many studies of NAc iGluRs following cocaine exposure have failed to detect long-term changes in NMDAR function (Bock et al., 2013; Kourrich et al., 2007; Thomas et al., 2001), while other more recent efforts have not addressed the question (Britt et al., 2012; Dobi et al., 2011; Mameli et al., 2009; Pascoli et al., 2014, 2012). To the best of

our knowledge, long-term functional changes in NAc NMDAR activity following cocaine exposure have never been published, and reports employing biochemical techniques have yielded mixed results (Mao et al., 2009; Schumann and Yaka, 2009). However, recent work from the lab of Woody Hopf has shown that the GluN2C-containing NMDARs pass current at hyperpolarized potentials following compulsive-like alcohol consumption (Seif et al., 2013). Genetic deletion of *Grin2C* was shown to eliminate compulsive alcohol intake, and further work has shown that pharmacological inhibition of these receptors recapitulates those behavioral effects (Seif et al., 2015).

Prior to (and perhaps facilitating) the long-term synaptic changes that occur following extended withdrawal is the generation of GluN2B-containing silent synapses (T. E. Brown et al., 2011; Huang et al., 2009a; Koya et al., 2012; Lee et al., 2013; Ma et al., 2014). Silent synapses, defined by the absence of functional AMPARs, are thought to be a substrate for increasing neuronal connectivity and may be important for drug-induced behavioral changes. Silent synapses are abundant in the developing brain, but their expression wanes as the brain matures and is considered to be minimal in adulthood. The primary direct measurement of silent synapses is known as a minimal stimulation assay. For this assay, a threshold intensity is selected such that approximately half of the stimuli evoke measureable AMPAR-EPSCs (~50% failure rate). The cell is then depolarized so NMDAR components can be observed, and the fraction of stimuli resulting in an EPSC is also measured. Populations containing many silent synapses will

generate relatively few NMDAR component failures, and the proportion of silent synapses in a neuron population can be subsequently calculated.

In a landmark paper, Huang et al. discovered that non-contingent cocaine administration generated an increase in NAc shell silent synapse function during subsequent short-term abstinence. The phenomenon peaked (~30% silent synapses) 3 days following the last cocaine exposure and returned to normal levels (~10%) 5-7 days later. Results from the minimal stimulation assay were confirmed by a ratiometric assessment of the coefficient of variation of the NMDAR component (Malenka and Nicoll, 1997). Furthermore, the increase in silent synapses was associated with enhanced GluN2B expression and function as assessed by Western Blot and sensitivity to the antagonist Ro 25-6981. Furthermore Brown et al. found that infusion of Ro 25-6981 into the NAc prevented the development of locomotor sensitization to cocaine. While not addressing D1/A2A cell type-specificity, others have demonstrated that these silent synapses are generated only in “neuronal ensembles” that express Fos following cocaine administration (Koya et al., 2012).

More recent experiments from the lab of Yan Dong have examined synaptogenesis in the context of self-administered cocaine. Lee et al., 2013, found that immediately following cocaine self-administration, BLA-NAc shell synapses display an enhanced proportion of silent synapses. As these synapses waned over time, and incubation of cocaine craving developed over time, a coincident upregulation of CP-AMPARs was observed. The authors then characterized BLA-specific NMDAR-dependent LFS LTD (3 min, 5 Hz), that selectively internalized

CP-AMPARs. When applied *in vivo*, the LTD eliminated incubation of cocaine craving, consistent with a direct role for CP-AMPARs in promoting that behavior.

Another study from the lab examined inputs from the PFC. Infralimbic (IL) projections to the NAc shell and prelimbic (PL) projections to the NAc core have been hypothesized to exert opposing effects on reinstatement and other drug-related behaviors (Peters et al., 2009). Therefore Ma et al. examined these two circuits in parallel in the context of cocaine self-administration. The authors observed increases in silent synapses at both IL-NAc shell and PL-NAc core synapses during short term abstinence, however at extended withdrawal CP-AMPARs were only observed at IL-NAc shell. LFS (10 min, 1 Hz) induced NMDAR- and $mGlu_1$-dependent LTD of CP-AMPARs at IL-NAc shell synapses, and enhanced the expression of incubation of cocaine craving when applied *in vivo*. In contrast, LFS induced NMDAR-dependent, $mGlu_1$-indepenent LTD of AMPARs at PL-NAc core synapses and inhibited cue-induced lever pressing.

Because MSNs *in vivo* reach stably depolarized plateaus following coincidental activity of inputs (O'Donnell and Grace, 1995), NAc synaptic NMDARs have been suggested to be activated without localized co-expression of AMPARs (Lee and Dong, 2011). Increases in GluN2B expression are also notable due to their increased conductance (Gielen et al., 2009; Paoletti et al., 2013) as well as enhanced coupling with downstream effectors such as calcium/calmodulin-dependent kinase II (Strack and Colbran, 1998; Strack et al., 2000). For these reasons, the transient upregulation of GluN2B-containng silent synapses has been proposed as a necessary mediator of long-term changes in NAc synaptic strength

following cocaine exposure (Lee and Dong, 2011). However, while the data provide more compelling evidence for the role of NAc AMPAR upregulation in the long-term behavioral effects of cocaine, the transient increase in silent synapses and GluN2B function remains largely coincidental. Aside from the NAc infusion of Ro 25-6981, none of these experiments have demonstrated the causal relationship between silent synapse function and the development of cocaine-conditioned behaviors. An alternative hypothesis, whereby GluN2B-containing NMDAR serve a feedback role to attenuating excessive drug-induced plasticity, remains equally plausible.

5. Summary

Addiction is clearly a complicated disease that recruits many neural circuits and intracellular signaling pathways. While this overview of addiction and current research strategies to study addiction-related phenomena has been broad, the complexities of the addicted brain are likely to depend on intricate temporal interactions between brain regions and signaling cascades. Nonetheless, it can be argued that addiction research has provided one of the most advanced understandings of experience-dependent plasticity, since models of addiction can be simplified as the effects of a substance (drugs of abuse) on a biological substrate (the brain). As such, addiction is one of the most powerful tools neuroscientists have to study learning and memory under controlled circumstances. As opposed to developmental or aging related diseases, exposure

to drugs of abuse is an inducible model under the temporal control of the experimenter.

The incorporation of cell type- and circuit-specific approaches to study neuroscience allow for rapid advancements in our understanding of addiction. Powerful tools including transgenic mice and optogenetics have revolutionized basic neuroscience research over the past decade (reviewed by Stamatakis and Stuber, 2012b). Optogenetic techniques have made it possible to dissect the function of different inputs to the VTA (Lammel et al., 2013, 2012) and NAc (Britt et al., 2012; Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014, 2012; Stuber et al., 2011) and in intact animals. These and similar approaches will continue to illuminate how synapses formed by these various inputs may be differentially modulated by drugs of abuse.

Prior to beginning my dissertation, few input-specific studies of excitatory synaptic physiology in the NAc had been reported in peer-reviewed journals. In three years, more than a dozen high-profile series of experiments have been published regarding NAc glutamatergic synaptic physiology (Britt et al., 2012; MacAskill et al., 2012) and pathophysiology in the context of alcohol (Seif et al., 2015, 2013) and cocaine (Creed et al., 2015; Lee et al., 2013; Ma et al., 2014; MacAskill et al., 2014; Pascoli et al., 2014, 2012; Stefanik et al., 2013; Suska et al., 2013; Terrier et al., 2015) abuse. Despite the rich interest in NAc synaptic physiology, these recent reports have by and large neglected to report on afferents from the mThal and how they may be modulated following drug exposure. Therefore we addressed this gap in knowledge with the aim of studying how

NMDAR function underlies cocaine-dependent alterations in NAc plasticity. We also generated and evaluated cell type-specific NMDAR genetic deletions to assess how striatal iGluR signaling contributes to reward-related behaviors. Our data support the leading hypotheses in the field, that NMDAR-dependent changes in D1 MSN function are requisite for long-term changes in physiology and behavior induced by cocaine exposure. However the work also provides the first characterization of mThal projections to the NAc and identifies GluN2C-containing NMDARs as novel mediators of cocaine-induced neuroplastic changes. By contrast A2A MSN NMDAR function may play a subtle role in reward-related behaviors, but was demonstrated to modulate behavioral despair. Together these data emphasize the divergent behavioral contributions of MSN cell types and identify the mThal-NAc pathway as potential target for ameliorating the persistent synaptic and behavioral changes brought about by cocaine.

CHAPTER III

METHODS AND MATERIALS

1. Mice

Bacterial artificial chromosome (BAC) adult (6–12 week) mice were used in all experiments and were housed together in groups of two-to-five per cage on a 12/12-hr light/dark cycle (lights on at 06:00), with food and water available *ad libitum*. Transgenic floxed GluN1 (*Grin1*$^{lox/lox}$) mice possess *loxP* sites flanking the transmembrane domain and C-terminal region (Tsien et al., 1996). Transgenic floxed Grin2B (*Grin2B*$^{lox/lox}$*)* mice were prepared as described (Brigman et al., 2010). *Grin1*$^{lox/lox}$ and *Grin2B*$^{lox/lox}$ mice were crossed with bacterial artificial chromosome (BAC) transgenic mice expressing Cre recombinase under the regulation of *Drd1a* (Gong et al., 2007; Nelson AB et al., 2012) and/or adenosine receptor subtype *Adora2a* (A2AR) promoters, and backcrossed to homozygosity of *Grin1*$^{lox/lox}$ *or Grin2B*$^{lox/lox}$. We elected to use A2AR as a marker for "indirect pathway" MSNs because A2AR is more selective than D2R, which is expressed by some striatal interneurons and as an autoreceptor on midbrain dopaminergic neurons. Mice used for electrophysiology also expressed tdTomato under the control of the *Drd1a* promoter (Gong et al., 2003; Shuen et al., 2008). Mice have been backcrossed onto a C57BL/6J background for >10 generations.

The open field test and cocaine conditioned place preference were performed in the Vanderbilt Mouse Neurobehavioral Core, where only male mice

were utilized. All other procedures were performed in the Grueter laboratory space. For the EPM, NIH, TST, and FST, both male and female mice were used. Sexes were housed, tested, and analyzed separately. Results generated from males and females were not statistically different, therefore the data was pooled and subsequently analyzed together. All experiments were done in accordance with the policies set out by the Institutional Animal Care and Use Committees (IACUC) at Vanderbilt University and in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2. Stereotaxic Injections

At 4-5 weeks of age, some mice underwent stereotaxic surgery for viral-mediated gene transfer of channelrhodopson-2-EYFP (ChR2). Mice were anesthetized with a dexmetomedine-ketamine mixture (0.5-80 mg/kg, *i.p.*). Bilateral 250-400 nL injections of AAV5-CaMKII-ChR2-EYFP (UNC Viral Vector Core) were delivered into the target region through a pulled glass pipette at 100 nL/min. Injection site coordinates were as follows (relative to bregma): mThal [ML: ± 0.3, AP: -1.2, DV: -3.0], PFC [ML: ± 0.3, AP: -1.75, DV: -2.75], BLA [ML: ± 3.1, AP: -1.25,

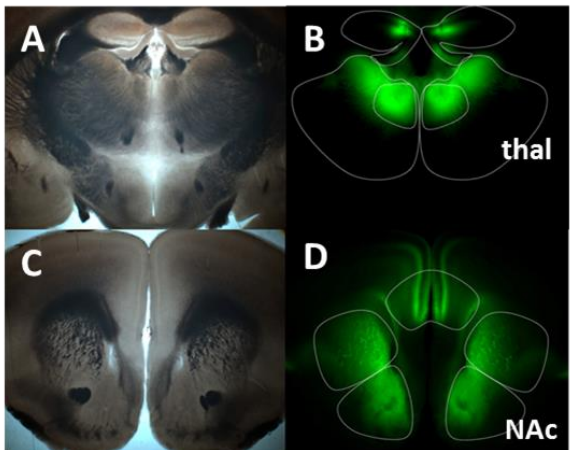


Figure 8. Representative AAV-ChR2-EYFP expression. (A) Coronal slice containing thalamus (thal) and hippocampus. (B) Robust EYFP expression is observed at the injection site. (C) Slice containing nucleus accumbens (NAc), dorsal striatum, and prefrontal cortex. (D) Expression of EYFP-labeled axon terminals is observed 4 weeks following viral-mediated gene transfer. AAV, adeno-associated virus; ChR2, channel-rhodopsin-2; EYFP, enhanced yellow fluorescent protein

DV: -4.75]. Atipamazole (1.0 mg/kg, *s.c.*) and ketoprofen (10 mg/kg, *s.c.*) were administered as a reversal agent and an analgesic. Mice were sacrificed for *ex vivo* electrophysiology 4-6 weeks following surgery to allow sufficient time for ChR2-EYFP expression in axon terminals within the NAc core and other regions innervated by the mThal (Figure 8, page 56).

3. Behavioral Procedures

3.1 Cocaine Conditioned Place Preference

3.1.1 *Grin1* Experiments

The place conditioning procedure was conducted in activity chambers identical to those used for the open field (ENV-510, Med Associates). Prior to the place conditioning pretest, mice were habituated to behavioral testing with a one-hour exposure to similar activity chambers. For place conditioning studies, a two-chambered insert with contextually distinct metal floors (ENV-3013-2, Med Associates, Figure 9) was placed in the activity chambers. All place conditioning sessions were 20 minutes in duration, and the amount of time spent in each zone was recorded. Conditioning sessions were conducted twice daily, separated by 4 hours, and treatment order was counterbalanced across groups. All mice received vehicle injections (0.9% saline, 10 µL/g, *i.p.*) prior to confinement in one compartment

Figure 9. Apparatus used for GluN1$^{-/-}$ conditioned place preference experiments. To minimize variability, cocaine was always paired with the side with bar floors (left) while saline was paired with the side with grid floors (right).

(mesh grid floor) and cocaine (20 mg/kg) immediately prior to confinement in the other compartment (parallel bar floor). Following the test of place preference expression, mice underwent extinction training. Subjects that did not express a place preference (<10%) were removed from the study. On days 1 and 3 of extinction, mice underwent saline conditioning sessions on both sides of the apparatus. One days 2 and 4 of extinction, standard test sessions occurred. Mice that did not adequately extinguish their place preference (>10%) on the second extinction test were removed prior to reinstatement. No difference in the proportion of mice removed from the study was observed across genotypes. To reinstate the place preference, mice received a lower priming dose of cocaine (10 mg/kg) immediately prior to the test session. Locomotor activity (distance traveled, stereotypic counts, vertical counts, and time at rest) was measured during all sessions.

3.1.2 *Grin2B* Experiments

These experiments were performed in the Grueter laboratory space in open field activity chambers (ENV-510, Med Associates). Overhead video recordings of mouse activity were analyzed with automated software (EthoVision XT, Noldus). The chamber was divided into two contextually distinct environments based on floor and wall (rough floor and vertical bar wall vs. smooth floor and checkerboard wall). Unlike the *Grin1* experiments, no innate preference for either side was consistently observed, therefore a predetermined design was not employed (i.e. cocaine was paired with the less-preferred side for each mouse). We assessed

sub-maximal cocaine CPP following one cocaine pairing, maximal cocaine CPP following 3 additional pairings, and maintenance of the reward-related memory at extended time points.

3.2 Open Field Assay

Mice were placed in an open field activity chamber (ENV-520, Med Associates) equipped with infrared beams and detectors for one hour, Each chamber was housed within a sound attenuating cabinet (ENV-022MD, Med Associates). A software interface (Activity Monitor, Med Associates) monitored the two-dimensional horizontal position of the mouse as well as beam breaks of a bar raised 4cm from the floor.

3.3 Elevated Plus Maze

The EPM was based on the model described by Lister (Lister, 1987). The EPM was comprised of two open and two closed arms (5 cm wide x 30 cm long) that meet in the center to form a plus. The floors were opaque and the walls were made of tinted black plastic. The arms of the EPM were elevated 40 cm above a platform. Animals were placed on an open platform facing the center and remained in the maze for 5 min. Visual recordings of mouse movement were obtained with an overhead video camera. Mouse location and movement were assessed in real time with automated software (EthoVision XT, Noldus). Subsequent analyses determined the duration of time the center-point of each mouse was in the open arms, closed arms, or center.

3.4 Novelty-induced Hypophagia

The NIH assay was based on a previously published procedure (Louderback et al., 2013). Briefly, mice were conditioned for 4 days to have limited (30 minutes) access to a tasty beverage (Ensure, home-made vanilla shake flavor) in their home cages while group housed. On the testing day, each mouse was transferred to an individual open field activity chamber and given access to Ensure for 30 minutes. The latency to drink and amount consumed throughout the session were measured.

3.5 Tail Suspension and Forced Swim Tests

The TST and FST were based on a previously published procedure (Lim et al., 2012). For the TST each mouse was suspended 20 cm above a surface with tape placed 1-2 cm from the base of the tail. For the FST, mice were placed in 2-L beakers half-filled with room temperature water that was changed between sessions. All trials were videotaped and scored by one-of-two trained observers. The interrater correlation (Pearson Product-Moment Correlation Coefficient, *r*) was calculated to be 0.978 (TST) and 0.941 (FST) by linear regression. Approximately 75% of analyses were performed blinded to genotype. Mice were considered immobile when, for more than 0.5 seconds, they: (TST) exhibited no body movement and hung passively or (FST) engaged in minimal movements to stay afloat with no escape behavior. Total immobility was scored as the sum of all immobility over the 6 minute trial. Latency was scored as the time of completion of the first uninterrupted 10-second bout of immobility.

3.6 Cocaine Conditioning

Mice were conditioned with injections of cocaine prior to electrophysiological recordings. Conditioning sessions began 2-3 weeks following surgery during the day phase of the light cycle. Following 2 days of habituation, mice underwent 5 daily injections with cocaine (15 mg/kg) or vehicle (Grueter et al., 2010; Kourrich et al., 2007; Thomas et al., 2001). Cocaine injections were administered immediately prior to placing the mouse in an open field activity chamber (ENV-510, Med Associates). Overhead video recordings of mouse activity were analyzed with automated software (EthoVision XT, Noldus).

4. Electrophysiology

4.1 Slice Preparation

Parasagittal slices (250 µm) containing the NAc core were prepared from naïve mice or from mice 10-14 days following the last cocaine/saline injection. Mice were anaesthetized with isoflurane and decapitated. Brains were quickly removed and placed in an ice-cold low-Na^+, sucrose-based cutting solution (in mM): 183 sucrose, 20 NaCl, 0.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$. Slices were prepared using a Leica VT 1200S vibratome and immediately transferred for 10-15 minutes to a heated (37 ± 1 °C) bath containing an N-methyl-D-glucamine (NMDG)-based recovery solution (in mM): 93 NMDG, 20 HEPES, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgCl_2$, 1.2 NaH_2PO_4, 25 glucose, 30 $NaHCO_3$, 5 Na-ascorbate, and 3 Na-pyruvate. Slices were then allowed to recover for at least 60 minutes at room temperature (23 ± 1 °C) in a holding chamber aCSF

(in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 11 glucose, and 26 $NaHCO_3$. Slices were removed from the holding chamber and placed in the recording chamber where they were continuously perfused with oxygenated (95% O_2 / 5% CO_2) aCSF at a rate of 2 ml/min at 30 ± 2°C. Picrotoxin (50 μM) was added to the recording aCSF to block $GABA_AR$–mediated inhibitory synaptic currents.

4.2 MSN Identification and Glutamate Release

Whole-cell voltage-clamp recordings were obtained as described (Grueter et al., 2013). Briefly, D1(+) MSNs were identified visually by the presence of tdTomato following excitation with 535 nm light (pE-2, CoolLED) (Figure 10). Neurons lacking any detectable tdTomato signal were defined as D1(-) MSNs rather than D2(+) because the absence of detectable fluorescence in this mouse line does not demonstrate that a cell is a MSN expressing D2Rs. Recordings were made with 3.0–6.0 MΩ glass electrodes pulled on a P-1000 Flaming/Brown puller (Sutter Instruments) filled with (in mM): 120 $CsMeSO_4$, 15 CsCl, 8 NaCl, 10

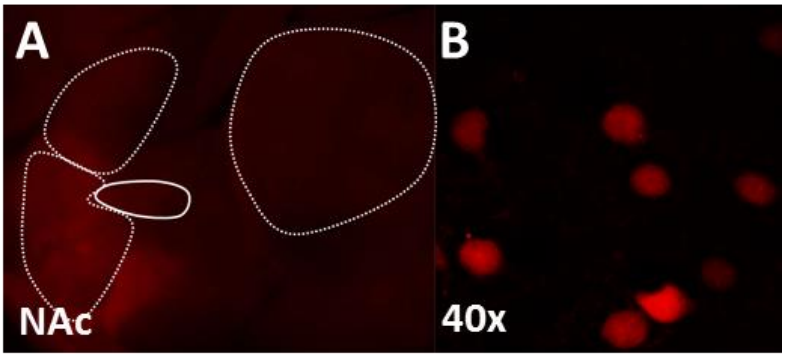


Figure 10. Representative D1-tdTomato expression. (A) Low magnification sagittal section displaying tdTomato expression in the dorsal striatum and nucleus accumbens (NAc). (B) High magnification image of the NAc core showing expression of tdTomato in cell bodies. Not pictured are D1(-) MSNs which do not express dopamine receptor subtype 1.

HEPES, 0.2 EGTA, 10 TEA-Cl, 4 MgATP, 0.3 NaGTP, 0.1 spermine, and 5 QX-314.

Where noted, excitatory afferents were locally stimulated (0.25-0.30 ms duration) with a bipolar nichrome wire electrode placed at the border between the NAc core and cortex. For optogenetic experiments, ChR2-dependent synaptic activity was driven using whole 470 nm field illumination for 1-3 ms. Recordings were performed using a Multiclamp 700B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz. Peak EPSC amplitudes of 100–600 pA were evoked at a frequency of 0.1-0.2 Hz while cells were voltage-clamped at -70 mV unless otherwise stated.

4.3 Experimental Protocols

For NMDAR/AMPAR ratios and coefficient of variation (CV) analyses, 25-30 stable consecutive responses at -70 mV and +40 mV were obtained. The NMDAR-component was defined as the magnitude of the +40 mV dual component EPSC at 50 ms following EPSC onset. The AMPAR-EPSC was defined as the peak amplitude of the EPSC at -70 mV. NMDAR/AMPAR was obtained by a ratio of the two components, and CV was calculated by dividing the standard deviation of the responses by the mean. To determine the current-voltage relationship and decay kinetics, NMDAR-EPSCs were isolated in the presence of 10 µM NBQX and corrections were made for the calculated liquid junction potential (-9.4 mV). For CIQ wash-on, NMDAR-EPSCs were elicited in CSF with no added Mg^{2+} (Weitlauf et al., 2004). Peak NMDAR-EPSC amplitudes ranged from 50-400 pA. For

asynchronous EPSC experiments, longer light durations were used (3-5 ms) and equimolar Sr^{2+} (2.5 mM $SrCl_2$) replaced Ca^{2+} in the aCSF. Asynchronous events were identified using a predefined template search in the 200 ms window following the initial evoked release event. NMDAR-dependent long-term depression was elicited by 600 pulses of 470 nm light at 1 Hz. Depolarization-induced suppression of excitation (DSE) was elicited by depolarizing to +30 mV for 10 seconds while patching with a modified potassium-based internal solution lacking BAPTA and QX-314 (Shonesy et al., 2013). Approximately 70% of experiments were performed blinded to treatment, however blinding to injection site was not possible. Data acquisition and analysis were performed using pClamp 10.4 software (Axon Instruments). Membrane properties were monitored continuously throughout the duration of experiments, and experiments in which changes in series resistance were greater than 20% were not included in the analysis.

5. Data Analysis

Data are expressed as mean ± SEM (N = number of animals and n = number of cells), or as [minimum, lower quartile, median, upper quartile, maximum] for box and whiskers plots. For electrophysiology each data point represents the average value from one cell. One- or two-way ANOVA or two-tailed Student's t-test were used when indicated. Repeated measures or paired tests were performed when appropriate. The Mantel-Cox log-rank test was used to assess differences between cumulative distributions. All post-tests employed Bonferroni

corrections for multiple comparisons and $p < 0.05$ was considered statistically significant.

6. Drugs

Cocaine HCl, picrotoxin, and d-cycloserine were purchased from Sigma-Aldrich. NBQX and D-AP5 were purchased from Tocris. (+)-CIQ was purchased from Brandt Labs.

CHAPTER IV

GLUN1 DELETIONS IN D1- AND A2A-EXPRESSING CELL TYPES REVEAL DISTINCT MODES OF BEHAVIORAL REGULATION

1. Abstract

NMDARs are profound regulators of glutamate neurotransmission and behavior. To coordinate components of the limbic system, the dorsal and ventral striatum integrate cognitive and emotional information towards the execution of complex behaviors. Striatal outflow is conveyed by MSNs, which can be dichotomized by expression of D1 or A2A. To examine how striatal NMDAR function modulates reward-related behaviors, we generated D1- and A2A-specific genetic deletions of the obligatory GluN1 subunit. Interestingly, we observed no differences in any GluN1$^{-/-}$ genotype in reward learning as assessed by acquisition or extinction of cocaine place conditioning. Control and A2A-GluN1$^{-/-}$ mice exhibited robust cocaine-primed reinstatement, however this behavior was markedly absent in D1-GluN1$^{-/-}$ mice. Interestingly, dual D1-/A2A-GluN1$^{-/-}$ mice displayed an intermediate reinstatement phenotype. Next, we examined models of exploration, anxiety, and despair, states often associated with relapse to addiction-related behavior, to determine NMDAR contribution in D1 and A2A cell types to these behaviors. D1-GluN1$^{-/-}$ mice displayed aberrant exploratory locomotion in a novel environment, but the phenotype was absent in dual D1/A2A-GluN1$^{-/-}$ mice. In contrast A2A-GluN1$^{-/-}$ mice displayed a despair-resistant phenotype, and this phenotype persisted in dual D1/A2A-GluN1$^{-/-}$ mice. These data support the

hypothesis that cell type-specific NMDAR signaling regulates separable behavioral outcomes related to locomotion, despair, and relapse.

2. Introduction

Adaptations involved in the development of addiction-like behaviors share common mechanisms with learning and memory processes (Grueter et al., 2012; Joffe et al., 2014; Koob and Volkow, 2010; Lüscher and Malenka, 2011). In particular, NMDARs have been extensively studied as mediators of drug experience-dependent memory formation (T. E. Brown et al., 2011; Kalivas and Alesdatter, 1993; Karler et al., 1989; Wolf and Khansa, 1991). NMDARs are glutamate-gated, calcium-permeable channels that provide major regulation of synaptic plasticity throughout much of the central nervous system (Traynelis et al., 2010), transforming transient patterns of neurotransmission into persistent changes in synaptic strength that underlie cognitive functions (Paoletti et al., 2013). Because constitutive genetic deletion of GluN1 is lethal (Tsien et al., 1996), probing NMDAR function in vivo necessitates cell type- and/or region-specific approaches.

One key brain complex that subserves reward-related behaviors is the striatum, which is conceptualized as a gatekeeper of descending neurotransmission relating to motor control, reward, and reinforcement (Grueter et al., 2012; Joffe et al., 2014; Kreitzer and Malenka, 2008). Throughout the dorsal and ventral striatum, GABAergic medium spiny neurons (MSNs) comprise 90-95% of neurons and provide the output from the structures. Striatal MSNs are often

dichotomized into two groups by biochemistry, anatomy, and function (Lobo and Nestler, 2011), commonly depicted by expression of D1 or A2A, which overlaps with D2-expressing MSNs. When selectively activated in vivo, D1 and A2A/D2 MSNs in the dorsal striatum and NAc have been shown to exert opposing or divergent effects on locomotor (Kravitz et al., 2010), stress-induced (Francis et al., 2014), and reward-related (Kravitz et al., 2012; Lobo et al., 2010) behaviors. For example, driving NAc D1 MSNs conferred CPP to a subthreshold cocaine regimen, whereas activation of NAc A2A/D2 MSNs blocked the preference induced by a rewarding dose.

Although much remains to be learned, NMDAR signaling in NAc D1 MSNs has been demonstrated to be necessary for the development of psychostimulant-conditioned behaviors (Beutler et al., 2011; Cahill et al., 2014; Heusner and Palmiter, 2005). However, these experiments did not assess whether D1-NMDAR signaling underlies reinstatement, a model of relapse to drug seeking. Additionally, how A2A MSN NMDARs modulate psychostimulant-conditioned behaviors has not been addressed. In fact, without limit to drug-related behaviors, the behavioral relevance of NMDAR signaling in A2A-expressing neurons remains unknown. Given that NMDAR signaling is important for experience-driven changes in D1 MSN function, a hypothesis is that A2A MSN NMDAR function regulates stress-related behaviors. Moreover, with recent efforts towards developing NMDAR antagonists and related pharmacotherapies as treatments for Major Depressive Disorder (Krystal et al., 2013; Maeng et al., 2008), the literature requires a better

understanding of how cell type-specific NMDAR function modulates despair- and anxiety-like behaviors.

The GluN1 subunit is essential to the formation of functioning NMDARs (Paoletti et al., 2013). Although splicing variants are common, all GluN1 protein is produced from a single gene, *Grin1*. Therefore we ablated functional NMDARs in D1- and/or A2A-expressing neurons by generating mice homozygous for floxed *Grin1* ($Grin^{lox/lox}$) that co-expressed Cre recombinase under control of the D1 and/or A2A promotors. MSN expression of D1 and A2A is by and large mutually exclusive (Gangarossa et al., 2013; Lu et al., 1998). Although D1 is expressed in other components of the limbic system, like the prefrontal cortex and amygdala (Boyson et al., 1986; Zhou et al., 1990), the neuronal expression of A2A is highly localized to the striatum (Fink et al., 1992; Schiffmann et al., 1991), where co-expression with D1 or interneuron markers is minimal (Schiffmann and Vanderhaeghen, 1993). Therefore, relative to the D1-specific deletion, the double knockout from D1- and A2A-expressing cell types induces identical impairments in NMDAR signaling across MSN types while exerting minimal additional extrastriatal off-target effects. After generating D1-, A2A-, and dual D1/A2A-$GluN1^{-/-}$ mice, we used targeted whole-cell electrophysiology to validate cell type specificity in the NAc core. We then assessed reward-related associative learning through cocaine CPP. All genotypes acquired, expressed, and extinguished cocaine CPP, however cocaine-primed reinstatement was markedly absent in D1-$GluN1^{-/-}$ mice. Dual D1-/A2A-$GluN1^{-/-}$ mice displayed a partial rescue, suggesting that both D1 MSNs and extrastriatal D1-expressing neurons may be involved in cocaine-primed

reinstatement. We then examined models of exploration, anxiety, and behavioral despair. D1-GluN1$^{-/-}$ mice displayed aberrant exploratory locomotion in a novel environment, but the phenotype was absent in dual D1/A2A-GluN1$^{-/-}$ mice. In contrast, A2A-GluN1$^{-/-}$ mice displayed an antidepressant-like phenotype that persisted in dual D1/A2A-GluN1$^{-/-}$ mice. These data suggest that balanced NMDAR signaling across striatal MSNs underlies aspects of locomotion, but that A2A MSN NMDAR function regulates despair in a more complex manner.

3. Results

3.1. Functional Verification of GluN1 Genetic Deletions

To confirm selectivity of the genetic deletions, we measured EPSCs in targeted NAc core MSNs from control animals which expressed either Cre or *Grin1*$^{lox/lox}$, but not both and D1-GluN1$^{-/-}$ and A2A- GluN1$^{-/-}$ mice. MSNs were identified as either D1-expressing (+) or D1-non-expressing (-) by presence or absence of td-Tomato fluorescence. In control MSNs held at -70 mV, the EPSC exhibits fast decay kinetics and is mediated by AMPARs (Figure 11A: left, page 71). When held at +40 mV, the dual component EPSC is much slower and at 50 ms post EPSC onset, reflects current passing solely through NMDARs. In control slices, measurable NMDAR currents (>20% of peak) were obtained in all NAc core MSNs (D1(+): n = 23/23, D1(-): n = 20/20). In contrast, in slices prepared from D1-GluN1$^{-/-}$ mice, NMDAR currents were obtained from D1(-) MSNs (n = 6/6) but not from D1(+) MSNs (n = 0/6, 5A: top right). In A2A-GluN1$^{-/-}$ mice, the reverse was observed: NMDAR currents were not observed in D1(-) MSNs (n = 0/11, 1A:

bottom right), but remained intact in D1(+) MSNs (n = 5/5). Fisher's exact test revealed a significant deviation from observed outcome frequencies across groups ($p < 0.001$ Figure 11B).

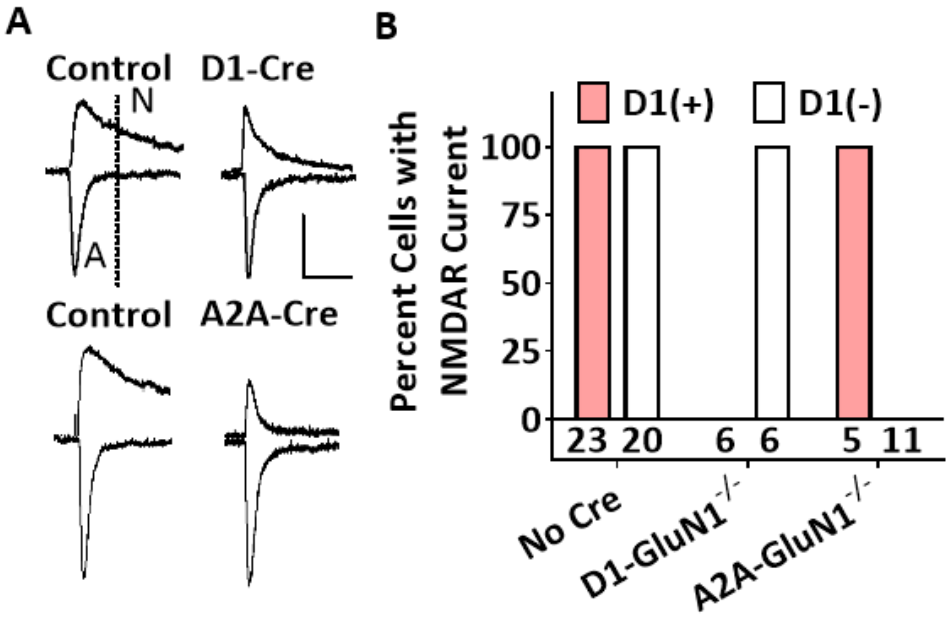


Figure 11. Selectivity of cell type-specific GluN1 deletions. (A) Representative traces of dual component EPSCs obtained from control (left) and GluN1$^{-/-}$ (right) NAc core MSNs. D1(+) MSNs are displayed on the top and D1(-) MSNs on the bottom. Scale bars denote 100 pA and 50 ms. The EPSC component at 50 ms or later is mediated by NMDARs. (B) Summary graph displaying percent D1(+) (red) and D1(-) (white) neurons with NMDAR currents ($p < 0.001$, Fisher's exact test). The number of cells examined is included under each bar.

3.2. Cocaine Place Conditioning in GluN1 Deletion Models

To determine the contribution of NMDAR signaling at D1- and A2A-cell types in the rewarding properties of cocaine, we performed cocaine place conditioning. During the conditioning sessions, locomotor activity was recorded. D1-GluN1$^{-/-}$ mice display diminished cocaine-induced hyperactivity (D1: 2837 ± 666 cm vs. control: 5170 ± 592 cm, $p < 0.05$, Figure 12), however the phenotype was absent in D1/A2A-GluN1$^{-/-}$ mice (5222 ± 704 cm,). All mice exhibited robust place preferences on the expression test day (Figures 13A-D), and no difference across genotypes was observed ($F(3,33) = 0.9733$, n.s.,).

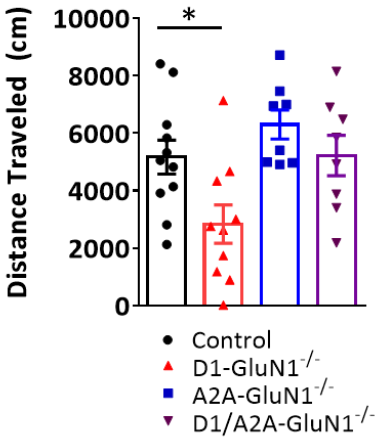


Figure 12. Locomotor response to cocaine. Distance traveled in the last cocaine conditioning session by control (black circles), D1-GluN1$^{-/-}$ (red triangles), A2A-GluN1$^{-/-}$ (blue squares), or D1/A2A-GluN1$^{-/-}$ (purple triangles) mice. D1-GluN1$^{-/-}$ exhibited attenuated locomotor response to cocaine relative to controls (different from control, *: $p<0.05$, Bonferroni post-test).

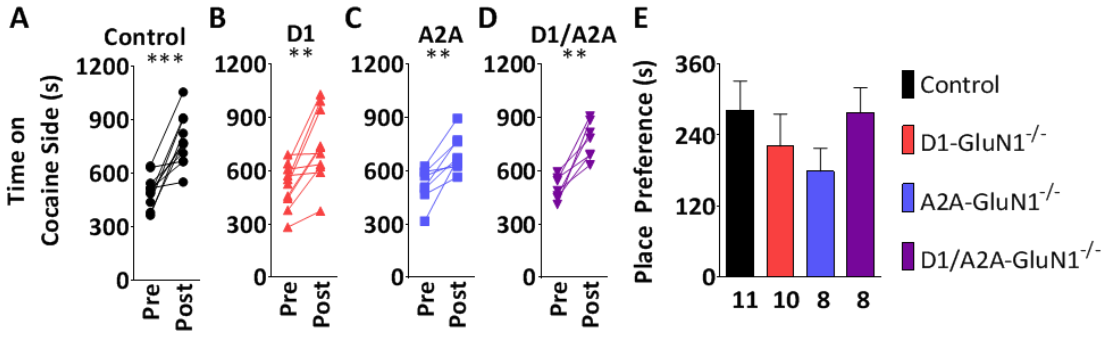


Figure 13. Expression of cocaine conditioned place preference. (A-D) Absolute time spent by control, D1-GluN1$^{-/-}$, A2A-GluN1$^{-/-}$, or D1/A2A-GluN1$^{-/-}$ mice on cocaine-paired side before (Pre) and after (Post) cocaine conditioning (**: $p < 0.01$, ***: $p < 0.001$, paired t-test). (E) Place preference following conditioning with 20 mg/kg *i.p.* cocaine in control (black), D1-GluN1$^{-/-}$ (red), A2A-GluN1$^{-/-}$ (blue), or D1/A2A-GluN1$^{-/-}$ (purple) mice.

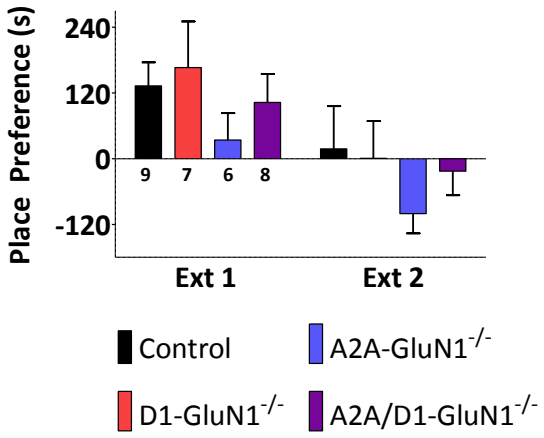


Figure 14. Extinction of cocaine conditioned place preference. The change in time spent on the cocaine-paired side was measured during a 4-day extinction protocol in control (black), D1-GluN1$^{-/-}$ (red), A2A-GluN1$^{-/-}$ (blue), or D1/A2A-GluN1$^{-/-}$ (purple) mice. No differences in extinction learning were observed.

Mice then underwent extinction training, and no differences were observed ($F(3,26) = 1.161$, n.s., Figure 14). Finally, to examine a facet of CPP related to relapse, mice were administered 10 mg/kg cocaine immediately prior to a place preference session. Control mice exhibited robust cocaine-primed reinstatement of CPP (432 ± 75 s, $p < 0.001$, Figure 15A, page 74). In contrast, D1-GluN1$^{-/-}$ mice exhibited a pronounced lack of reinstatement (-5 ± 136 s, n.s, Figure 15B.). Like the controls, the A2A-GluN1$^{-/-}$ mice reinstated the place preference (463 ± 54 s, $p < 0.001$, Figure 10C), and the D1/A2A-GluN1$^{-/-}$ mice also displayed intact cocaine-induced reinstatement (274 ± 62 s, $p < 0.01$, Figure 15D), rescuing the phenotype displayed by the D1-GluN1$^{-/-}$ animals. Although the D1/A2A-GluN1$^{-/-}$ mice displayed cocaine-primed reinstatement, the magnitude of the preference was less relative to the control group so we performed a between-groups analysis (Figure 15E). A one-way ANOVA revealed a main effect of genotype on reinstatement ($F(3,22) = 10.17$, $p < 0.001$). Subsequent Bonferonni post-tests against controls confirmed the significant impairment of reinstatement in D1-GluN1$^{-/-}$ mice ($t = 4.550$, $p < 0.001$) and also revealed a blunted phenotype in the D1/A2A-GluN1$^{-/-}$ group ($t = 2.698$, $p < 0.05$). This suggests that drug-primed reinstatement of

cocaine CPP requires NMDARs in D1-expressing cells, and that balanced striatal signaling is, at most, influential over the behavior.

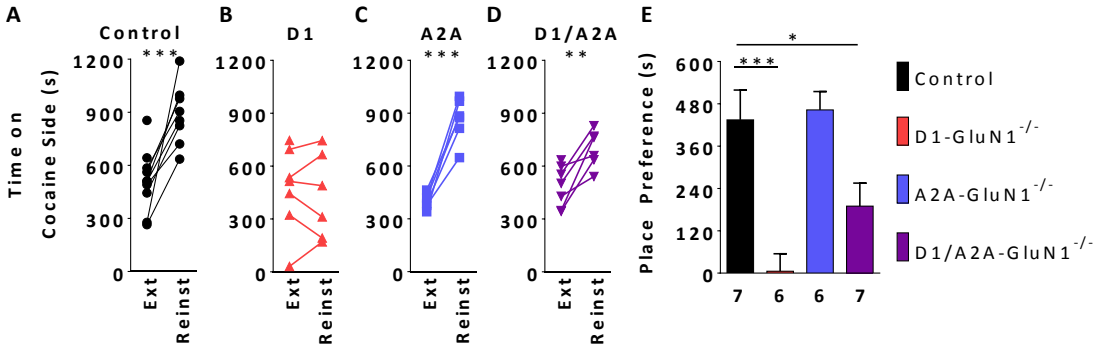


Figure 15. Reinstatement of cocaine conditioned place preference. (**A-D**) Absolute time spent by WT, D1-GluN1$^{-/-}$, A2A-GluN1$^{-/-}$, or D1/A2A-GluN1$^{-/-}$ mice on cocaine-paired side after extinction (Ext) and priming with 10 mg/kg cocaine (Reinst) (**: $p < 0.01$, ***: $p < 0.001$, paired t-test). Data points without paired reinstatement values represent mice that did not meet extinction criterion and were subsequently excluded. (**E**) Place preference in control (black), D1-GluN1$^{-/-}$ (red), A2A-GluN1$^{-/-}$ (blue), or D1/A2A-GluN1$^{-/-}$ (purple) mice following reinstatement primed by 10 mg/kg *i.p.* cocaine (different from control, *: $p < 0.05$, ***: $p < 0.001$, Bonferroni post-test).

3.3. Open Field Test in GluN1 Deletion Models

To examine a range of locomotor-based behaviors, we assessed spontaneous activity in an open field chamber. When placed in a novel environment, mice display a burst of horizontal locomotor activity during the first 5-10 minutes that declines substantially over time ($F(11,374) = 34.06$, $p < 0.0001$, Figure 16A, page 75). A two-way repeated measures ANOVA revealed a significant interaction between the locomotor time course and genotype ($F(33,374) = 6.818$, $p < 0.0001$). Post-tests determined that D1-GluN1$^{-/-}$ mice exhibited attenuated novelty-induced hyperlocomotion (0-5 min, 553 ± 95 cm vs. control: 1139 ± 140 cm). On the other hand, this behavior remained intact in A2A-GluN1$^{-/-}$ mice. D1-GluN1$^{-/-}$ mice also displayed atypical habituation to the novel

environment, as evidenced by elevated locomotor activity towards the end of the hour session (50-55 min, 532 ± 55 cm vs. control: 238 ± 77 cm). Both of these phenotypes were rescued in the D1/A2A-GluN1$^{-/-}$ mice, suggesting that imbalanced striatal signaling underlies the D1-GluN1$^{-/-}$ locomotor phenotypes. When summated over the entire hour-long test, no differences in total locomotion were observed ($F(3,34) = 1.244$, n.s., Figure 16B).

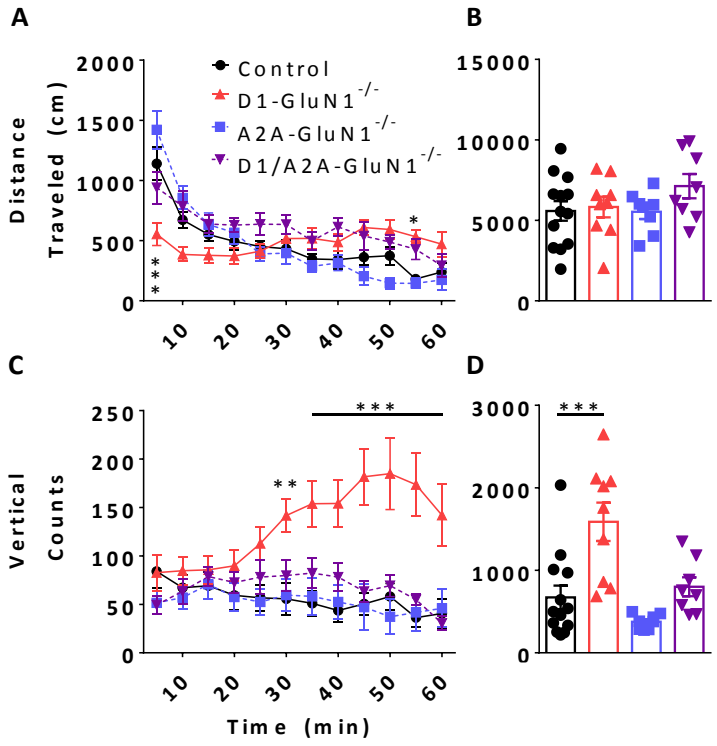


Figure 16. Open field assay. (A) Novelty-induced hyperactivity and habituation in control (black circles), D1-GluN1$^{-/-}$ (red triangles), A2A-GluN1$^{-/-}$ (blue squares), or D1/A2A-GluN1$^{-/-}$ (purple triangles) mice (different from control, *: $p<0.05$, ***: $p<0.001$, Bonferroni post-test). (B) Total one-hour locomotor activity in control (black), D1-GluN1$^{-/-}$ (light grey), A2A-GluN1$^{-/-}$ (dark grey), or D1/A2A-GluN1$^{-/-}$ (white) mice. (C) Vertical beam breaks binned over 5 minute periods (different from control, **: $p<0.01$, ***: $p<0.001$, Bonferroni post-test). (D) Total vertical beam breaks obtained during the open field assay (different from control, ***: $p<0.001$, Bonferroni post-test).

During the same open field session, vertical beam breaks were also measured, having been described previously in relation to exploratory behavior (Fonio et al., 2009). We found that vertical counts varied significantly by time ($F(11,374) = 2.455$, $p < 0.01$, genotype ($F(3,34) = 6.681$, $p < 0.01$), and an interaction ($F(33,374) = 5.657$, $p < 0.0001$). Subsequent analyses revealed that D1-GluN1$^{-/-}$ mice displayed increased vertical counts during the second half of the hour session (Figure 16C). The increase in vertical counts is also evident when summated across the entire session (D1: 1588 ± 698 vs control: 673 ± 143, $p < 0.001$, Figure 16D). Similar to the aberrations in horizontal locomotion, the vertical count phenotype was rescued by the D1/A2A-GluN1$^{-/-}$ manipulation, without significant contribution of the A2A-specific knockout alone. Together these data suggest that D1-NMDAR function can modulate locomotor activity by disrupting the balance of signaling across MSN cell types.

We assessed the time spent in the center of the chamber during the open field test and found significant differences across genotypes ($F(3,34) = 4.050$, $p < 0.05$, Figure 17A, page 77). Control and D1-GluN1$^{-/-}$ mice both spent approximately 35% and 29% of the session in the center zone, respectively (control: 1260 ± 151 s, D1: 1034 ± 184 s). In contrast, A2A-GluN1$^{-/-}$ mice displayed increased thigmotactic behavior and spent only 18% of the session in the center zone (643 ± 46s, $p < 0.05$). Like the altered locomotor behaviors in the D1-GluN1$^{-/-}$ mice, the center time phenotype of the A2A-GluN1$^{-/-}$ was absent in the D1/A2A-GluN1$^{-/-}$ mice (37%, 1340 ± 149 s). Because decreases in center time often reflect increased anxiety, we hypothesized that A2A-GluN1$^{-/-}$ mice display a high-anxiety-

like phenotype and proceeded to assess anxiety- and depressive-like behaviors in more targeted assays.

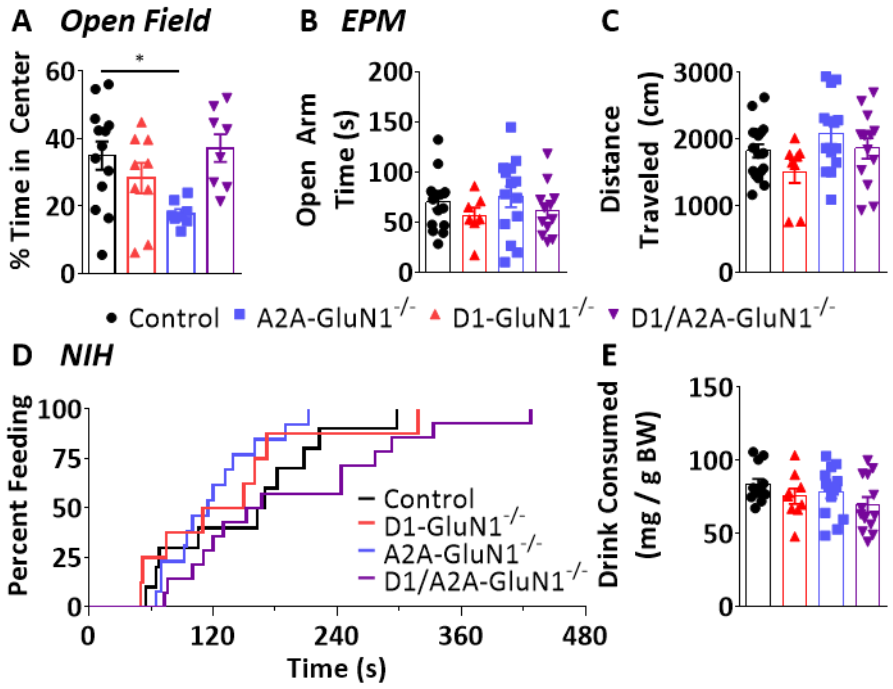


Figure 17. Anxiety-like behavior assays. (A) Time spent in center of arena during first open field assay in control (black circles), D1-GluN1$^{-/-}$ (red triangles), A2A-GluN1$^{-/-}$ (blue squares), or D1/A2A-GluN1$^{-/-}$ (purple triangles) mice (different from control, *: $p<0.05$, Bonferroni post-test). (B) Time spent in open arms during elevated plus maze (EPM). (C) Total distance traveled during elevated maze session. (D) Cumulative distribution of latency to consume palatable drink during novelty-induced hypophagia (NIH) test session. (E) Total amount of drink consumed (normalized to bodyweight) during test session.

3.4. Elevated Plus Maze and Novelty-induced Hypophagia in GluN1 Deletion Models

To examine anxiety-like behavior, we first utilized the elevated plus maze (EPM). The EPM takes advantage of the innate tendency of rodents to avoid open spaces. No significant differences in open arm time during the EPM session were observed across genotypes ($F(3,45) = 0.8393$, n.s.) (Figure 17B): control and all

GluN1$^{-/-}$ mice also spent similar amounts of time in the closed arms (58%, control: 176 ± 7 s, D1: 192 ± 11 s, A2A: 164 ± 15 s, D1/A2A: 188 ± 6 s; $F(3,45) = 1.386$, n.s.; data not shown). No significant differences in locomotor activity during the EPM session were observed across genotypes ($F(3,45) = 2.163$, n.s., Figure 17C). As a complimentary assessment of anxiety-like behavior, we examined novelty-induced hypophagia (NIH). We employed a version of NIH in which non-food restricted mice must approach a highly palatable drink in a novel environment, and the latency to consume the liquid is taken as a measure of anxiety-like behavior. A Mantel-Cox log-rank test determined that control and all GluN1$^{-/-}$ mice exhibited similar latency distributions ($\chi^2 = 5.281$, df = 3, n.s., Figure 17D). Furthermore no difference in mean latency (control: 164 ± 22 s, D1: 136 ± 31s, A2A: 120 ± 13s, D1/A2A: 196 ± 29 s; $F(3,43) = 2.119$, n.s.) or overall drink consumption was observed ($F(3,43) = 1.617$, n.s., Figure 17E).

3.5. Tail Suspension and Forced Swim Tests in GluN1 Deletion Models

We performed the tail suspension and forced swim tests, models of behavioral despair. In each of these tests, mice attempt to remove themselves from compromising, yet inescapable positions. The animals eventually assume an immobile posture, and the latency to hang/float is taken as a behavioral correlate of despair. No differences in immobility between controls and any GluN1$^{-/-}$ mice were observed in the TST when measured in total ($F(3,69) = 2.129$, n.s., Figure 18A). However a significant effect of genotype on the latency to immobility was observed ($F(3,67) = 5.415$, $p < 0.05$, Figure 18B), and post-tests revealed an increased mean latency in the A2A-GluN1$^{-/-}$ mice (181 ± 20 s vs control: 100 ± 9 s, $p < 0.01$).

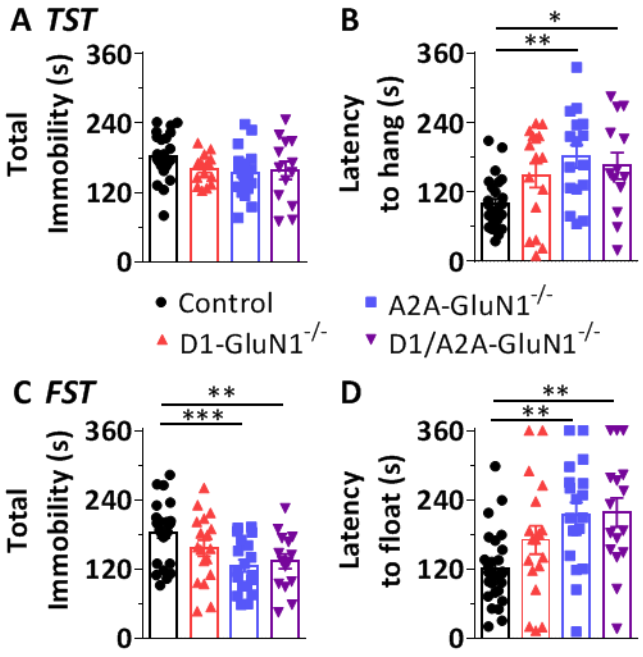


Figure 18. Behavioral despair assays. (A) Total time spent immobile during tail suspension test (TST) in control (black circles), D1-GluN1$^{-/-}$ (red triangles), A2A-GluN1$^{-/-}$ (blue squares), or D1/A2A-GluN1$^{-/-}$ (purple triangles) mice. (B) Latency to hang immobile for 10 s (different from control, *: $p<0.05$, **: $p<0.01$, Bonferroni post-test). (C) Total time spent immobile during forced swim test (FST) (different from control, **: $p<0.01$, ***: $p<0.001$, Bonferroni post-test). (D) Latency to float immobile for 10 s (different from control, **: $p<0.01$ Bonferroni post-test).

Under conditions of greater duress in the FST (Solich et al., 2008), significant genotypic differences in total immobility were observed ($F(3,75) =$

5.722, $p < 0.01$, Figure 18C). Specifically, A2A-GluN1$^{-/-}$ mice exhibited a decrease in total immobility (125 ± 10 s vs control: 183 ± 10 s, $p < 0.001$) consistent with an increased mean latency to float (214 ± 21 s vs control: 121 ± 12 s, $p < 0.01$, Figure 18D). In each of these tests and measures, D1-GluN1$^{-/-}$ mice did not differ from controls (TST total: 161 ± 6 s, latency: 149 ± 21 s; FST total: 156 ± 14 s, latency: 170 ± 25 s). Interestingly, D1/A2A-GluN1$^{-/-}$ mice behaved similarly to A2A-GluN1$^{-/-}$ mice and different from controls on all measures. That is, restoring striatal NMDAR balance did not rescue the A2A-GluN1$^{-/-}$ phenotype: D1/A2A-GluN1$^{-/-}$ mice exhibited an increased latency to hang during the TST (165 ± 23 s), $p < 0.05$ and to float during the FST (218 ± 25 s, $p < 0.01$), as well as decreased total immobility during the FST (133 ± 13 s, $p < 0.01$).

4. Discussion

Striatal NMDAR signaling has been implicated in the pathophysiology of drug addiction and other psychiatric diseases with motivational or affective components (Beutler et al., 2011; Cahill et al., 2014; Lim et al., 2012; Pascoli et al., 2014; Schwartz et al., 2014). In this report, we demonstrate separable roles for cell type-specific NMDAR function in the regulation of complex behaviors. These data support the hypothesis that balanced signaling across MSN subtypes regulates some locomotor-dependent behaviors, while providing further evidence that a more complex model is required to describe reward-related and depressive-like outcomes.

We observed no effect of any genotype on the acquisition of cocaine CPP. These findings may appear to disagree with published literature, where similar impairments of NMDAR function resulted in blunted psychostimulant CPP (Beutler et al., 2011; Heusner and Palmiter, 2005), however specific methodological differences (i.e. background strain, apparatus, and drug) can readily explain minor discrepancies. To summarize, the published accounts and present data collectively demonstrate that a moderate dose of cocaine does not require D1-NMDAR function to generate reward learning. We did observe that D1-GluN1$^{-/-}$ mice exhibited a stark impairment of drug-primed reinstatement of cocaine CPP. These data are in accordance with recent findings, where putative NMDAR-dependent modifications on NAc D1 MSNs were demonstrated to be essential for cue-induced reinstatement of cocaine seeking (Pascoli et al., 2014). Our data corroborate the mechanistic underpinnings of those findings and support the importance of D1 MSN NMDARs in drug-primed reinstatement. Moreover we observed that dual D1/A2A-GluN1$^{-/-}$ mice retained cocaine-primed reinstatement, suggesting that balanced NMDAR signaling across striatal projection neurons may underlie this phenomenon. On the other hand, the dual knockout mice did exhibit blunted reinstatement relative to control mice, suggesting that NMDARs in extrastriatal D1-expressing neurons may contribute to drug-primed reinstatement. Consistently, Sanchez and Sorg (Sanchez et al., 2003) demonstrated that reinstatement of cocaine CPP was blocked by infusion of a D1 antagonist into the PFC. D1-expressing neurons in the orbitofrontal cortex (Capriles et al., 2003;

Lasseter et al., 2013) and amygdala (Alleweireldt et al., 2006; S. Erb et al., 2001; Mashhoon et al., 2009) represent other intriguing candidate substrates.

To assess movement-based adaptive learning, we examined locomotor activity in a novel environment. D1-GluN1$^{-/-}$ mice displayed a substantial attenuation of hyperactivity during the first five minutes, and did not habituate normally as evidenced by increased activity after 50 minutes. During the same task, D1-GluN1$^{-/-}$ mice displayed a robust increase in vertical counts, which have been described as a form of exploratory behavior (Fonio et al., 2009). Neither of these locomotor phenotypes were present in D1/A2A-GluN1$^{-/-}$ mice, supporting the conclusion that balanced MSN NMDAR signaling regulates exploration and habituation. We also observed that A2A-GluN1$^{-/-}$ mice spent less time in the center of the arena than controls. Since thigmotactic behavior can be indicative of an anxiety-like phenotype, we followed up with more targeted assays. Contrary to our working hypothesis, A2A-GluN1$^{-/-}$ mice performed similarly to controls on both the EPM and NIH. We therefore conclude that A2A-GluN1$^{-/-}$ mice display atypical patterns of horizontal locomotion, and hypothesize that more rigorous assessments of movement (for example, see Paulus and Geyer, 1991) would reveal subtle differences in unconditioned locomotion.

Since depressive symptoms are often comorbid with drug abuse and addiction (Joffe et al., 2014; Volkow, 2010), we assessed behavioral despair in two parallel assays, the TST and the FST. Due to previously observed genotype-sex interactions in these behaviors (Kokras and Dalla, 2014) we ran separate cohorts of male and female mice. We observed similar results in both sexes: A2A-GluN1^{-}

$^{/-}$ mice displayed a prolonged latency to immobility in both assays and increased total immobility in the FST. These findings suggest that A2A-GluN1$^{-/-}$ mice exhibit a despair-resistant or "resilient" phenotype. Ionotropic glutamate receptor signaling in the NAc has been heavily implicated in depressive-like behaviors (Bagot et al., 2015; Francis et al., 2014; Lim et al., 2012; Robison et al., 2014; Schwartz et al., 2014; Vialou et al., 2010). Our data suggest that these characterized physiological processes may proceed largely through A2A MSNs, consistent with recent findings that the development of amotivation induced by chronic pain proceeds through NMDAR signaling on NAc A2A MSNs (Schwartz et al., 2014). Together these data support the hypothesis that A2A MSN NMDARs signaling promotes depressive-like behavior. Conversely, the antidepressant efficacy of fluoxetine requires downregulation of the NMDAR signaling partner, CaMKII (Robison et al., 2005; Shonesy et al., 2014). Whether changes to A2A MSN NMDAR signaling are required for the behavioral efficacy of SSRIs and other antidepressants remains untested and merits further study. Interestingly D1/A2A-GluN1$^{-/-}$ mice also displayed the anti-despair phenotype exhibited by A2A-GluN1$^{-/-}$ mice. Unlike the locomotor-based assays, these results suggest that A2A MSNs contribute to despair-like behavior in a manner that cannot be distilled to balanced MSN NMDAR signaling. One intriguing possibility is that A2A MSNs, or their targets, project to a region outside of the canonical basal ganglia that modulates negative affective behaviors.

If indeed striatal balance is required for the expression of some psychostimulant and novelty-related behaviors, it begs the question: why did A2A-

GluN1$^{-/-}$ mice not display unusual drug-related behaviors? We propose two, non-exclusive explanations. For one, from a practical perspective, we may have encountered ceiling effects in the selected classical conditioning paradigms. Operant behavioral paradigms would be expected to provide a greater signal window for detecting increases in drug reinforcement and susceptibility to relapse. Accordingly, following chemogenetic inhibition of NAc A2A MSNs, Bock et al. observed enhanced motivation to obtain cocaine as assessed by lever-pressing behavior. Alternatively, following a sub-threshold training protocol or priming dose, A2A-GluN1$^{-/-}$ mice might display or reinstate CPP under conditions where control mice would not.

CHAPTER V

COCAINE EXPERIENCE ENHANCES THALAMO-ACCUMBENS N-METHYL-D-ASPARTATE RECEPTOR FUNCTION

1. Abstract

Excitatory synaptic transmission in the NAc is a key biological substrate underlying behavioral responses to psychostimulants and susceptibility to relapse. Recent studies have demonstrated that cocaine induces changes in glutamatergic signaling at distinct inputs to the NAc. However, consequences of cocaine experience on synaptic transmission from the mThal to the NAc have yet to be reported. To examine synapses from specific NAc core inputs, we recorded light-evoked EPSCs following viral-mediated expression of ChR2 in the mThal, PFC, or BLA from acute brain slices. To identify NAc MSN subtypes we utilized mice expressing tdTomato driven by the promoter for the D1. We recorded NMDAR and AMPAR properties to evaluate synaptic adaptations induced by cocaine experience, a 5-day cocaine exposure followed by 2-weeks of abstinence. We determine that excitatory inputs to the NAc core display differential NMDAR properties. Furthermore, cocaine experience uniquely alters AMPAR and NMDAR properties at mThal-D1(+), mThal-D1(-), and PFC-D1(+) synapses, while sparing PFC-D1(-) synapses. Finally, at mThal-D1(+) synapses, we demonstrate that cocaine enhances GluN2C/D function and NMDAR-dependent synaptic plasticity.

Our results identify contrasting cocaine-induced AMPAR and NMDAR modifications at mThal- and PFC-NAc core synapses. These changes include an enhancement of NMDAR function and plasticity at mThal-D1(+) synapses. Incorporation of GluN2C-containing NMDARs most likely underlies these phenomena and represents a potential therapeutic target for psychostimulant use disorders.

2. Introduction

Addressing the persistent vulnerability of relapse to drug-seeking behavior remains an essential obstacle impeding the treatment of substance abuse disorders (Joffe et al., 2014). The long-lasting urges and maladaptive behaviors conditioned by cocaine use are associated with changes in excitatory transmission throughout the mesolimbic dopamine system, including within the NAc (Grueter et al., 2012; Robison and Nestler, 2011). Recent studies have supported the hypothesis that alterations in NAc AMPAR signaling are essential towards promoting maladaptive drug-associated behaviors (Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014, 2012).

Seminal findings by Thomas et al (Thomas et al., 2001) demonstrated that AMPAR function in the NAc shell is altered by cocaine history. Since then, we and others have demonstrated circuit-specific, experience-dependent changes in NAc synaptic function by utilizing reporter mouse lines that label neuron populations and optogenetic approaches to drive specific excitatory afferents. The output neurons of the NAc, MSNs, are largely distinguished by expression of D1 or D2.

While both D1 (Bock et al., 2013; Dobi et al., 2011; Jedynak et al., 2015; Khibnik et al., 2015; MacAskill et al., 2014; Pascoli et al., 2014, 2012) and D2 (Bock et al., 2013; Grueter et al., 2010; MacAskill et al., 2014) NAc MSN subtypes have been shown to undergo drug-induced changes in AMPAR function, these changes occur in an experience-, subregion-, and input-specific manner. In contrast to AMPARs, drug-induced changes in NAc NMDAR function have been less well documented. Following a short-term abstinence from cocaine exposure Huang et al, and others, have reported a transient increase in the expression of silent synapses (T. E. Brown et al., 2011; Huang et al., 2009b; Koya et al., 2012; Lee et al., 2013; Ma et al., 2014), synapses that exhibit robust NMDAR-mediated EPSCs but undetectable AMPAR-EPSCs (Kerchner and Nicoll, 2008; Malenka and Nicoll, 1997). These silent synapses are thought to be due in large part to the insertion of NMDARs containing the GluN2B subunit. Conversely, at 14 days following chronic amphetamine, decreased expression of GluN2B-containing NMDARs has been reported (Mao et al., 2009). Also, longer-lasting increases in NAc hyperpolarization-active GluN2C-containing NMDARs are implicated in alcohol seeking (Seif et al., 2015, 2013), but their role in cocaine-related experience is unclear. Since NMDAR subunit composition can have a profound effect on synaptic function, the molecular stoichiometry of NMDARs in drug- and synapse-specific models warrants further investigation.

The NAc core and shell receive excitatory input from the PFC, BLA, and the vHipp (Britt et al., 2012; Brog et al., 1993; O'Donnell and Grace, 1995). Dysregulated plasticity of AMPARs has been identified as a substrate that

promotes reward-driven and addiction-like behaviors at NAc synapses from each of these inputs (Britt et al., 2012; Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014, 2012). Projections from the mThal also innervate the NAc (Berendse and Groenewegen, 1990; Vertes et al., 2015), yet whether drug experience alters these synapses remains to be shown.

The thalamus is described as a sensorimotor-cortical relay, and has received relatively little attention with regards to its capacity to modulate complex affective and cognitive behaviors (Vertes et al., 2015). The thalamic PVT and paratenial nucleus receive inputs from multiple brain regions implicated in addiction-related behavior and project to the NAc (Berendse and Groenewegen, 1990; Li and Kirouac, 2012; Phillipson, 1988). Thus the mThal nuclei are anatomically positioned to provide essential coordination of learned drug-induced behaviors. Indeed, lesion or pharmacological inhibition of the PVT has been demonstrated to disrupt NAc-dependent behaviors including reinstatement of cocaine-seeking and expression of cocaine conditioned place preference (Browning et al., 2014; Hamlin et al., 2008; James et al., 2010; Young and Deutch, 1998). Therefore, to begin to address the role of mThal-NAc pathways in drug-related behaviors, we sought to test the hypothesis that cocaine exposure alters mThal-NAc AMPAR and NMDAR function.

To better understand mThal inputs to NAc core we compared AMPAR and NMDAR properties at mThal, PFC, and BLA synapses. We expressed ChR2 in BAC transgenic D1-tdTomato mice and performed targeted, input-specific, whole-cell voltage-clamp recordings in acute slices of the NAc core. Synaptic

NMDAR/AMPAR ratios differed based on glutamatergic input. At mThal-D1(+) synapses, cocaine experience, defined as abstinence from 5 days of cocaine exposure, enhanced AMPAR function and altered NMDAR properties, while at mThal-D1(-) synapses the cocaine-induced changes were consistent with generation of silent synapses. In contrast to mThal-NAc synapses, inputs from the PFC to NAc core D1(+) MSNs, but not D1(-) MSNs, exhibited larger NMDAR/AMPAR ratios following cocaine experience. Finally, we found that cocaine experience enhanced GluN2C/D function and unmasked NMDAR-dependent long-term depression (LTD) at mThal-D1(+) synapses. These results suggest that cocaine experience generates changes in NMDAR function at mThal-NAc core synapses and identifies these adaptations as targets with therapeutic potential for the treatment of psychiatric disorders associated with dysfunction of the reward system.

3. Results

3.1 Circuit-specific AMPAR and NMDAR Properties of NAc Core MSNs

To characterize synaptic AMPAR and NMDAR properties in the NAc core in a circuit-specific manner, we virally expressed ChR2 in the mThal, PFC, or BLA of D1-tdTomato marker mice. After 3 weeks robust expression of ChR2 was observed at the injection site and in the NAc (Figure 19A, page 91). Utilizing visualized whole-cell voltage-clamp techniques, we recorded light-evoked EPSCs at D1(+) and D1(-) MSNs. To compare AMPAR function across NAc core inputs, we elicited asEPSCs in low-Ca^{2+} aCSF supplemented with Sr^{2+}. asEPSCs are

quantal-like events that reflect synaptic expression of AMPARs. The asEPSC amplitudes were similar across inputs onto D1(+) (Figure 19B1) and D1(-) (Figure 19B2) MSNs.

To better understand mThal-NAc core synaptic composition, we compared NMDAR/AMPAR ratios to those recorded following PFC and BLA stimulation. The NMDAR-mediated component was recorded at 50 ms post onset (when the AMPAR component is minimal) and normalized to the AMPAR EPSC peak amplitude for each cell (Grueter et al., 2010; Kreitzer and Malenka, 2007). mThal synapses on D1(+) and D1(-) cell types exhibited smaller NMDAR/AMPAR ratios relative to the PFC (mThal-D1(+): 0.325 ± 0.029 vs. PFC-D1(+): 0.564 ± 0.066, $p < 0.01$, Figure 19C1; mThal-D1(-): 0.322 ± 0.029 vs. PFC-D1(-): 0.576 ± 0.042, $p < 0.001$, Figure 19C2). D1(-) synapses from the BLA also exhibited smaller NMDAR/AMPAR ratios relative to the PFC (BLA-D1(-): 0.372 ± 0.043, $p < 0.01$).

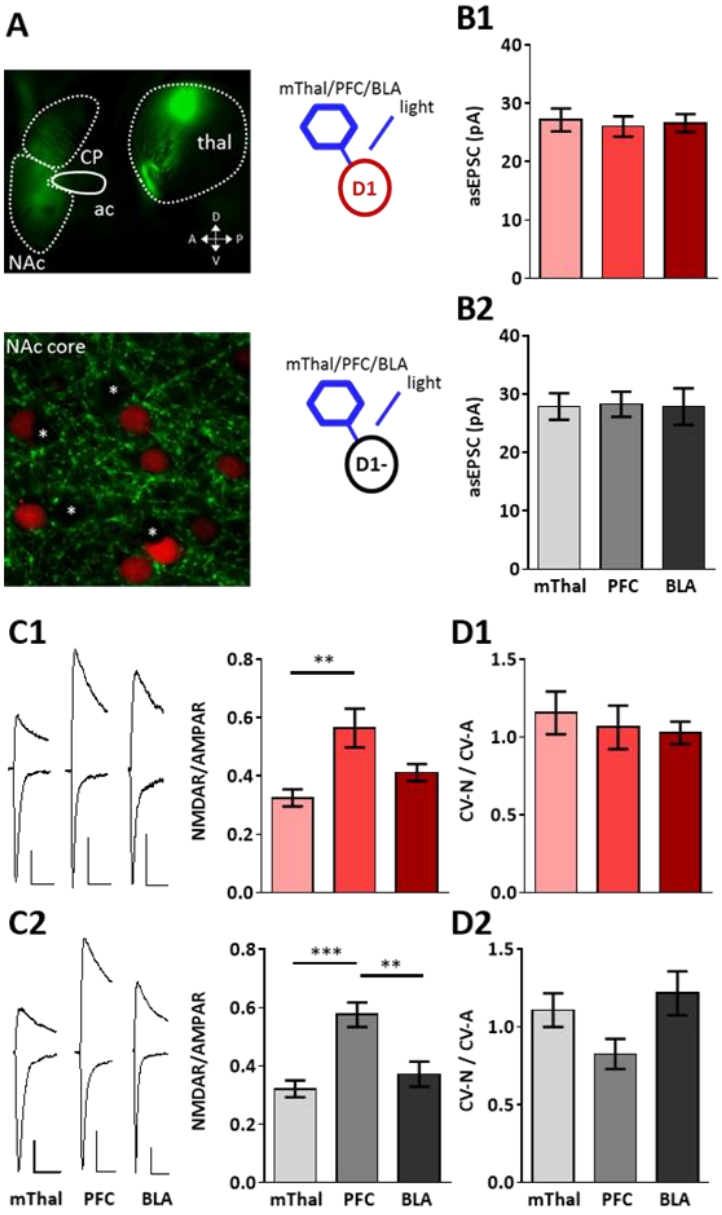


Figure 19. Basal AMPAR and NMDAR properties of NAc core synapses. (A) ChR2-EYFP expression in D1-tdTomato brain slice following viral-mediated gene transfer directed at the mThal. Wide field sagittal section (top) and high magnification merged confocal image (bottom) are displayed. (B-D) Input-specific AMPAR and NMDAR properties onto D1(+/-) neurons: (B) Amplitude of asEPSCs at D1(+) (B1) and D1(-) (B2) synapses following stimulation of terminals from the mThal (light red/grey), PFC (medium red/grey) or BLA (dark red/grey) in the presence of Sr^{2+}. (n = 7-9 / N = 4 per group). (C) Ratio of NMDAR component relative to AMPAR EPSC while recording from D1(+) (C1) and D1(-) (C2) neurons. Representative input-specific EPSCs at -70 mV and +40 mV are displayed on the left. Inputs from mThal exhibited lower NMDAR/AMPAR than from the PFC (**: $p < 0.01$; ***: $p < 0.001$, post-tests). (n = 10-13 / N = 5-10 per group). (D) Silent synapse CV analysis. CV-NMDAR normalized to CV-AMPAR at D1(+) (D1) and D1(-) (D2) synapses.

As mentioned above, silent synapses are putative substrates for long-term potentiation that are characterized by the functional presence of NMDARs but not AMPARs (Kerchner and Nicoll, 2008; Malenka and Nicoll, 1997). Silent synapses are abundant in the developing brain, and recent work has demonstrated that cocaine exposure transiently increases silent synapses in the NAc (T. E. Brown et al., 2011; Huang et al., 2009b; Koya et al., 2012; Lee et al., 2013; Ma et al., 2014). Therefore, we analyzed CV-N/CV-A, a measure inversely related to the proportion of silent synapses (Huang et al., 2009b; Kullmann, 1994). We found no difference in CV-N/CV-A across inputs onto D1(+) (Figure 19D1) or D1(-) MSNs (Figure 19D2). Analysis of CV measurements in isolation can be found at the end of this chapter. Taken together, these data are consistent with the interpretation that glutamatergic inputs to the NAc core display basal differences in NMDAR function. This led us to assess whether cocaine experience would alter NMDAR function at these specific NAc core synapses.

3.2 Cocaine Experience Does Not Alter Electrically-evoked NAc core AMPAR or NMDAR Properties

In order to investigate consequences of cocaine experience on NAc synaptic properties we conditioned mice with 5-days of cocaine injections outside of the home cage. Repeated injections of cocaine induced robust increases in locomotor activity ($F = 27.46$, $df = 6$, $p < 0.001$, Figure 20A, page 93). Following a 10-14 day drug-free period, we performed targeted whole-cell recordings of electrically-evoked EPSCs in the NAc core. We observed no effects of this cocaine

conditioning paradigm on NMDAR/AMPAR ratios or properties within the NAc core on either D1(+) (Figure 20B1-E1) or D1(-) MSNs (Figure 20B2-E2).

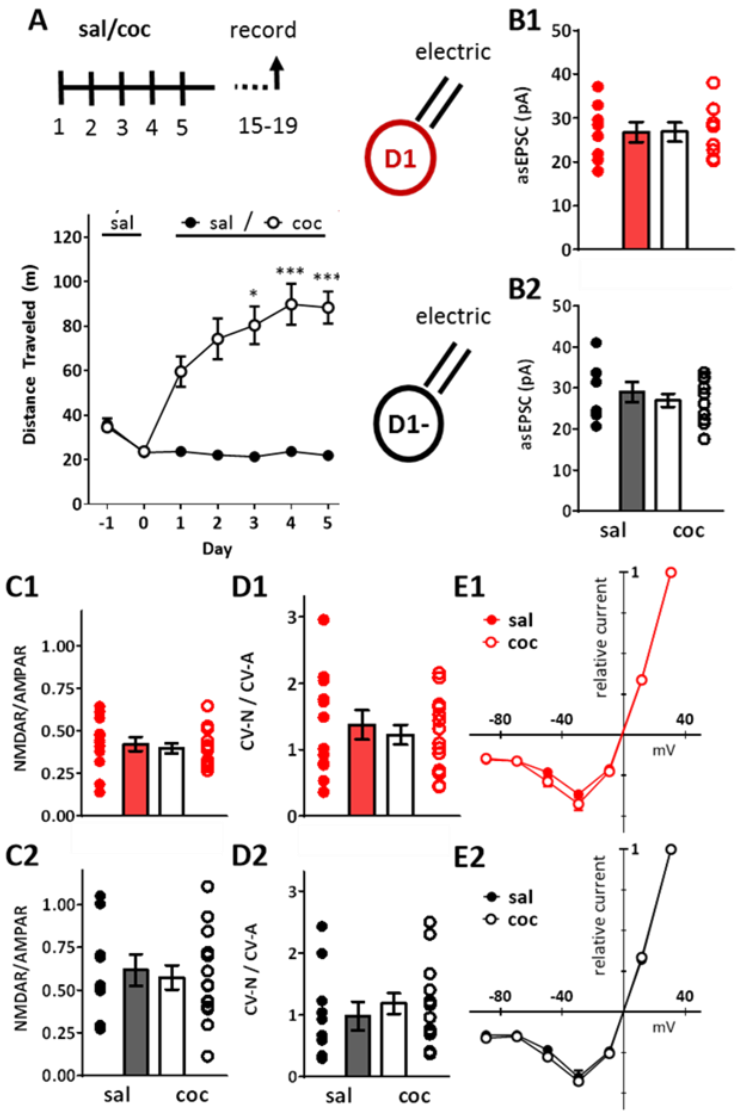


Figure 20. Cocaine exposure and abstinence does not affect electrically-evoked NAc core NMDAR or AMPAR function. (A) Top, schematic of behavioral conditioning and targeted approach. Slices were prepared 10-14 days after mice were conditioned with saline (filled circles) or cocaine (open circles) and EPSCs were elicited with a local electrode. Bottom, mice exhibited a sensitized locomotor response to cocaine (*: $p < 0.05$; ***: $p < 0.001$, post-test vs. day 1). (N = 20 saline; 16 cocaine). (B) Amplitude of asEPSCs from both D1(+) (B1) and D1(-) (B2) neurons. (n = 7-9 / N = 4-6 per group). (C) Ratio of NMDAR component to AMPAR EPSC following while recording from D1(+) (C1) or D1(-) (C2) neurons. (n = 9-14 / N = 7-9 per group). (D) CV-NMDAR normalized to CV-AMPAR in D1(+) (D1) and D1(-) (D2) cell types. (E) Isolated NMDAR current-voltage relationship from both D1(+) (E1) and D1(-) (E2) neurons. (n = 8-16 / N = 5-8 per group).

3.3 Cocaine Experience Alters AMPAR and NMDAR Function at mThal-D1(+) NAc Core Synapses

To investigate potential changes in excitatory drive specific to thalamic inputs following cocaine experience, we examined AMPAR and NMDAR function at mThal-D1(+) NAc core synapses (Figure 21A, page 95). We observed that cocaine experience increased the amplitude of AMPAR-mediated asEPSCs at these synapses relative to saline controls (sal: 23.9 ± 1.6 pA vs coc: 31.2 ± 2.6 pA, $p < 0.05$, Figure 21B). We expected that cocaine experience would result in a corresponding decrease in NMDAR/AMPAR. However mThal-D1(+) NMDAR/AMPAR at mThal-D1(+) from cocaine-treated mice was indistinguishable from saline controls (Figure 21C). Also, no change in CV-N/CV-A between the cocaine- and saline-treated groups was observed (Figure 21D). Given that we observed a cocaine-induced increase in AMPAR function, we hypothesized that cocaine treatment would be associated with a concomitant increase in NMDAR function at these synapses, and proceeded to analyze pharmacologically-isolated NMDAR EPSCs (Figure 21E). The current-voltage relationship of NMDAR EPSCs is generally determined by the biophysical properties of GluN2 subunits expressed at the synapse (Paoletti et al., 2013). After normalizing to the peak NMDAR-EPSC at +30 mV, we observed that cocaine exposure increased the relative mThal-D1(+) NMDAR current passed at -30 mV (sal: -0.334 ± 0.057 vs coc: -0.465 ± 0.041, $p < 0.05$). This change in the current-voltage relationship is consistent with the interpretation that cocaine experience altered the functional subunit stoichiometry of mThal-D1(+) NMDARs, potentially by increasing the expression of GluN2C

isoforms (Kuner and Schoepfer, 1996). Overall, mice with a cocaine history exhibited a potentiation of synaptic strength/connectivity at mThal-D1(+) synapses, despite no observed change in the NMDAR/AMPAR ratio.

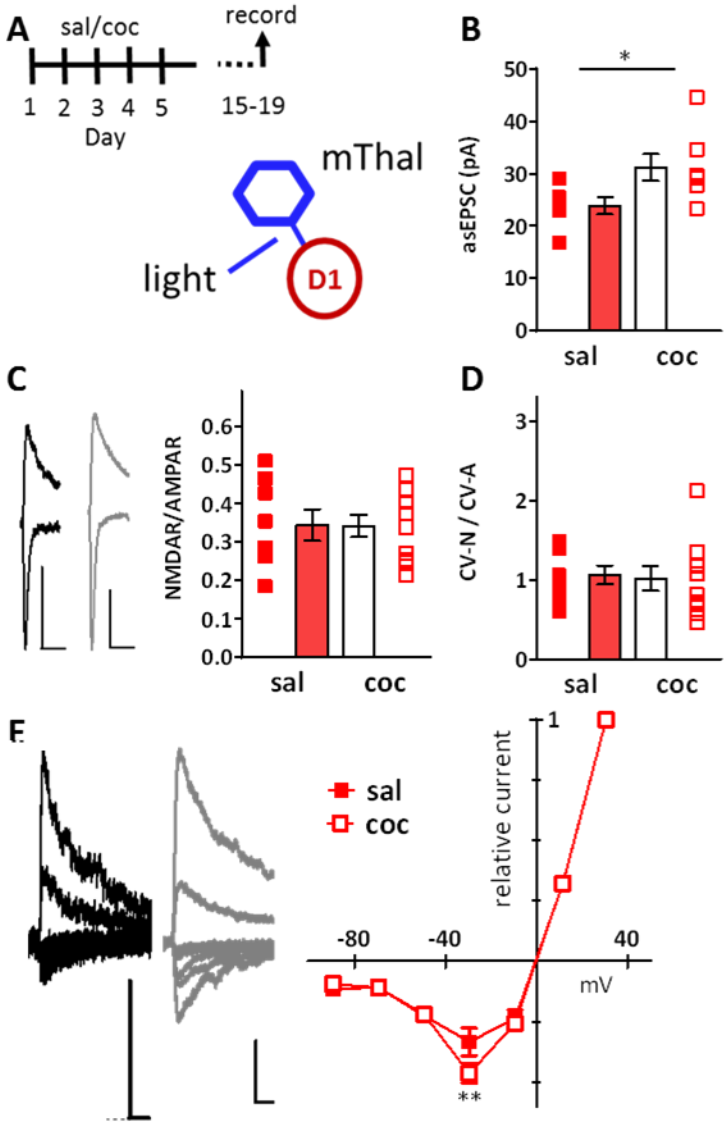


Figure 21. Cocaine enhances mThal-D1(+) AMPAR and NMDAR function. (A) D1(+) neurons were targeted in slices prepared 10-14 days after mice were conditioned with saline (filled squares) or cocaine (open squares), and glutamate was released from the mThal. (B) Cocaine conditioning increased the amplitude of asEPSCs at mThal-D1(+) NAc core synapses ($p < 0.05$). (n/N = 6/4 saline; 7/4 cocaine). (C) Left, dual component EPSCs obtained at -70 mV and +40 mV in slices from mice conditioned with saline (black) or cocaine (grey) mice. The NMDAR component is normalized to the AMPAR EPSC. Scale bars denote 100 pA and 50 ms. Right, summary data of NMDAR/AMPAR ratios. (n/N = 8/8 saline; 10/10 cocaine). (D) CV-NMDAR normalized to CV-AMPAR. (E) Left, isolated NMDAR current-voltage relationship. Scale bars denote 50 pA and 50 ms. Right, mThal-D1(+) NAc core synapses exhibited an altered NMDAR current-voltage relationship following in vivo cocaine conditioning (**: $p < 0.01$). (n/N = 6/6 saline; 9/7 cocaine).

3.4 NMDAR Function at PFC-D1(+) NAc Core Synapses Is Increased Following Cocaine Experience

To probe how cocaine experience affects cortical inputs to the NAc core, we examined AMPAR and NMDAR properties utilizing ChR2 expressed in axon terminals from the PFC (Figure 22A, page 97). In contrast to mThal inputs, we found no difference in asEPSC amplitude between cocaine- and saline-treated mice (Figure 16B). However, PFC-D1(+) NMDAR/AMPAR ratio was greater following cocaine experience relative to the saline-treated group (sal: 0.388 ± 0.040 vs coc 0.550 ± 0.067, $p < 0.05$, Figure 22C). To determine whether silent synapses may contribute to the increase in NMDAR/AMPAR ratio we analyzed CV-N/CV-A, but found no difference between cocaine and vehicle groups (Figure 22D). Finally, we examined NMDAR biophysical properties to address potential differences in subunit stoichiometry across groups. We also found no differences in current-voltage relationship (Figure 22E) suggesting that NMDAR subunit stoichiometry at PFC-D1(+) inputs is not modified by cocaine experience. Together these data indicate that at PFC-D1(+) NAc core synapses, cocaine experience yields an upregulation of NMDAR function without increasing silent synapses or altering subunit stoichiometry.

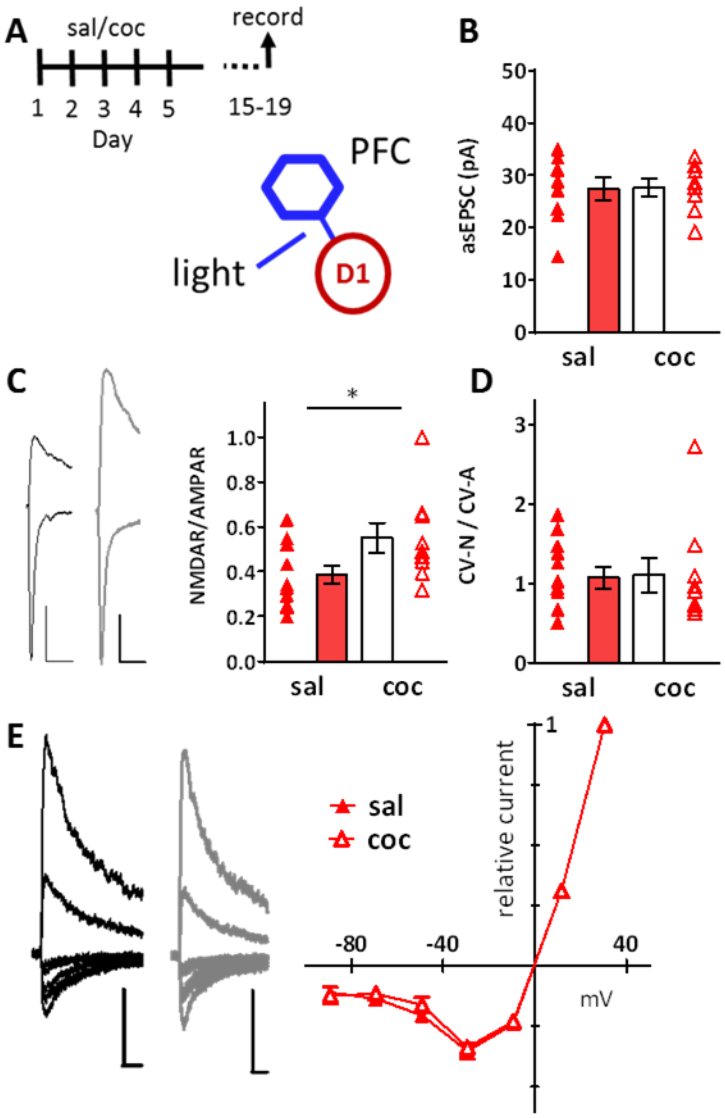


Figure 22. Cocaine experience enhances PFC-D1(+) NMDAR function. (A) D1(+) neurons were targeted in slices prepared 10-14 days after mice were conditioned with saline (filled triangles) or cocaine (open triangles), and glutamate was released from the PFC. (B) asEPSC amplitude of PFC-D1(+) synapses. (n/N = 9/4 saline; 8/4 cocaine). (C) Left, dual component EPSCs obtained at -70 mV and +40 mV. Scale bars denote 100 pA and 50 ms. Right, cocaine exposure and abstinence increased NMDAR/AMPAR relative to saline controls ($p < 0.05$). (n/N = 12/6 saline; 9/5 cocaine). (D) CV-NMDAR normalized to CV-AMPAR. (E) Left, isolated NMDAR current-voltage

3.5 mThal-D1(-) NAc Core Synapses Exhibit Functional Properties Consistent with More Silent Synapses Following Cocaine Experience

In parallel to recordings from D1(+) MSNs, we assessed consequences of cocaine experience on AMPAR and NMDAR properties at mThal-D1(-) synapses (Figure 23A, page 99). The asEPSC amplitude did not differ between cocaine and saline treatment groups (Figure 23B). However unlike mThal-D1(+), NMDAR/AMPAR at mThal-D1(-) synapses was larger in the cocaine-treated group (sal: 0.309 ± 0.045 vs coc: 0.422 ± 0.024, $p < 0.05$, Figure 23C). Furthermore, we observed a much smaller CV-N/CV-A ratio following cocaine experience (sal: 1.224 ± 0.167 vs coc: 0.609 ± 0.050, $p < 0.01$, Figure 23D). Cocaine experience did not alter mThal-D1(-) NMDAR stoichiometry as measured by current-voltage relationship (Figure 23E), however silent synapses are not known to be enriched in NMDARs with abnormal current-voltage relationships.

3.6 Cocaine Experience Does Not Alter PFC-D1(-) NAc Core AMPAR or NMDAR Properties

We also examined whether cocaine treatment induced changes in AMPAR or NMDAR function at PFC-D1(-) NAc core synapses. No differences in asEPSC amplitude, NMDAR/AMPAR ratiometric measures, or NMDAR current-voltage relationship at PFC-D1(-) synapses (Figure 24A-E, page 100) were observed following cocaine experience.

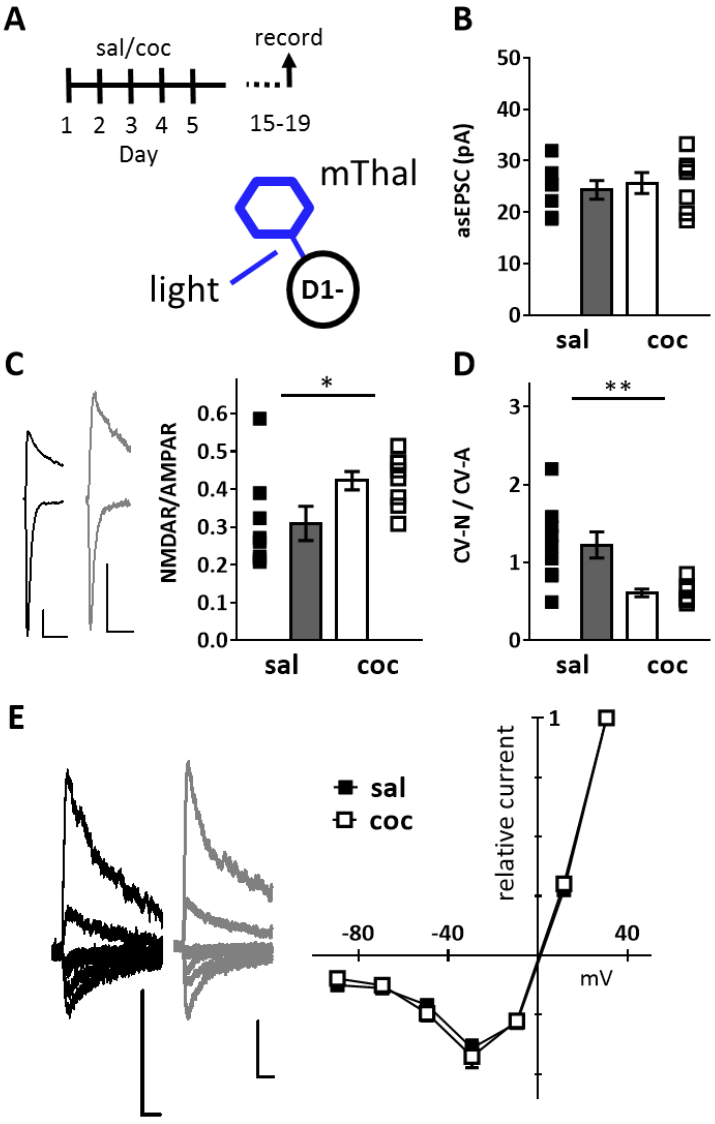


Figure 23. Cocaine history induces changes consistent with more silent synapses at mThal-D1(-) synapses in NAc core. (A) D1(-) neurons were targeted in slices prepared 10-14 days after mice were conditioned with saline (filled squares) or cocaine (open squares), and glutamate was released from the mThal. (B) Amplitude of asEPSCs elicited in Sr^{2+}-replaced aCSF. (n/N = 7/4 saline; 7/4 cocaine). (C) Left, the NMDAR component is normalized to the AMPAR-EPSC. Scale bars denote 100 pA and 50 ms. Right, cocaine exposure increased NMDAR/AMPAR at mThal-D1(-) synapses ($p < 0.05$). (n/N = 8/8 saline; 8/8 cocaine). (D) CV-NMDAR (CV-N) normalized to CV-AMPAR (CV-A). Cocaine conditioning decreased CV-N/CV-A at mThal-D1(-) NAc core synapses ($p < 0.01$). (E) Left, isolated NMDAR current-voltage relationship. Scale bars denote 50 pA and 50 ms. Right, summary data of NMDAR current-voltage relationships. (n/N = 7/5 saline; 7/4 cocaine).

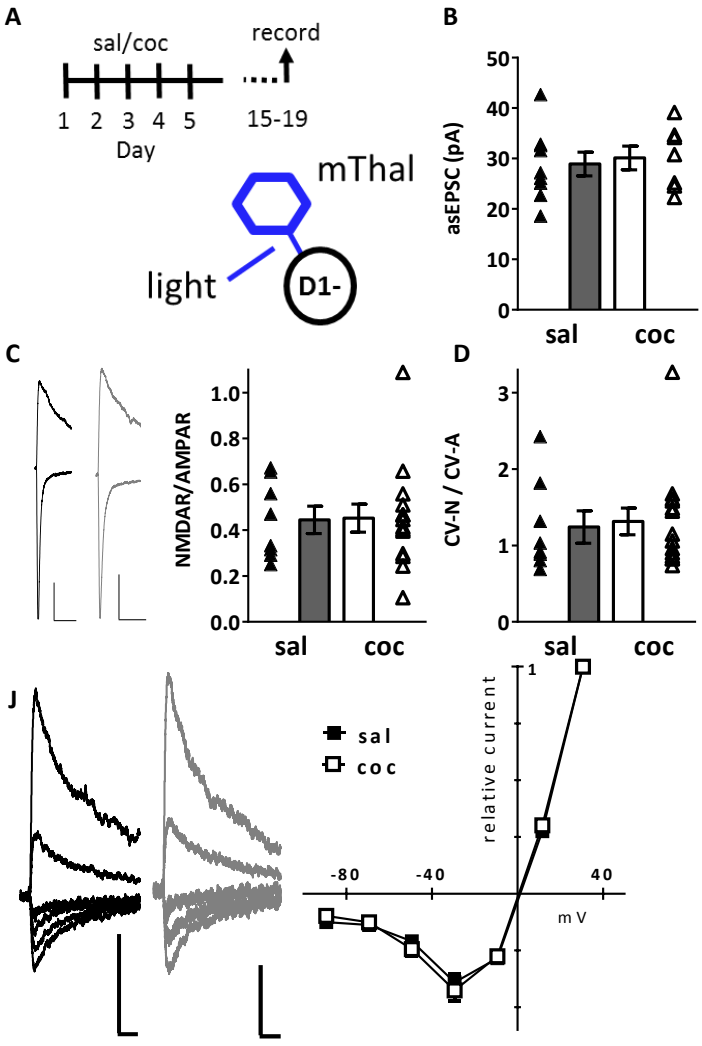


Figure 24. Cocaine experience does not alter PFC-D1(-) synapses. (A) D1(-) neurons were targeted in slices prepared 10-14 days after mice were conditioned with saline (filled triangles) or cocaine (open triangles), and glutamate was released from PFC. (B) asEPSC amplitude of PFC-D1(-) synapses. (n/N = 9/4 saline; 7/4 cocaine). (C) Left, dual component EPSCs obtained at -70 mV and +40 mV. Scale bars denote 100 pA and 50 ms. Right, summary data of NMDAR/AMPAR ratios. (n/N = 8/5 saline; 14/8 cocaine). (D) CV-NMDAR normalized to CV-AMPAR. (E) Left, isolated NMDAR current-voltage relationship. Scale bars denote 50 pA and 50 ms. Right, summary data of NMDAR current-voltage relationships. (n/N = 6/3 saline; 5/3 cocaine).

3.7 Functional Upregulation of GluN2C/D at mThal-D1(+) Synapses Following Cocaine Experience

In order to investigate the cocaine-induced changes in NMDAR current-voltage relationship, we utilized the positive allosteric modulator CIQ (Mullasseril et al., 2010; Ogden et al., 2014) to probe GluN2C/2D function. Washing on CIQ exerted no effect on the size of the mThal AMPAR EPSCs on D1 MSNs from either cocaine-treated mice or controls (Figure 25B, page 102), so we proceeded to assess isolated NMDAR currents in low-Mg^{2+} aCSF containing NBQX (Figure 20C). Application of CIQ did not affect mThal-D1(+) NMDAR-EPSCs from control animals (96.0 ± 9.2%, Figure 25D), but potentiated NMDAR currents at mThal-D1(+) synapses from cocaine-conditioned mice (130.3 ± 12.0%, $p < 0.05$ vs. sal).

To confirm that the effect of CIQ occurs in standard, Mg^{2+}-containing aCSF, we examined NMDAR/AMPAR ratios in a separate set of slices (Figure 25E). In control D1 MSNs, mThal NMDAR/AMPAR was not affected by the presence of CIQ (Figure 25F). However CIQ generated an increase in mThal NMDAR/AMPAR in D1 MSNs from cocaine-conditioned animals, (aCSF: 0.394 ± 0.030 vs CIQ: 0.531 ± 0.044, $p < 0.05$). To further investigate the contribution of GluN2C-containing NMDARs, we isolated NMDAR-EPSCs in the presence of d-cycloserine, a GluN2C-preferring agonist (Dravid et al., 2010; Seif et al., 2015; Sheinin et al., 2001). Bath application of d-cycloserine abrogated the cocaine-induced alteration in NMDAR current-voltage relationship at mThal-D1(+) synapses (Figure 26, page 103). The findings from these convergent

pharmacological experiments are consistent with the interpretation that cocaine exposure enhanced the function of GluN2C-containing NMDARs.

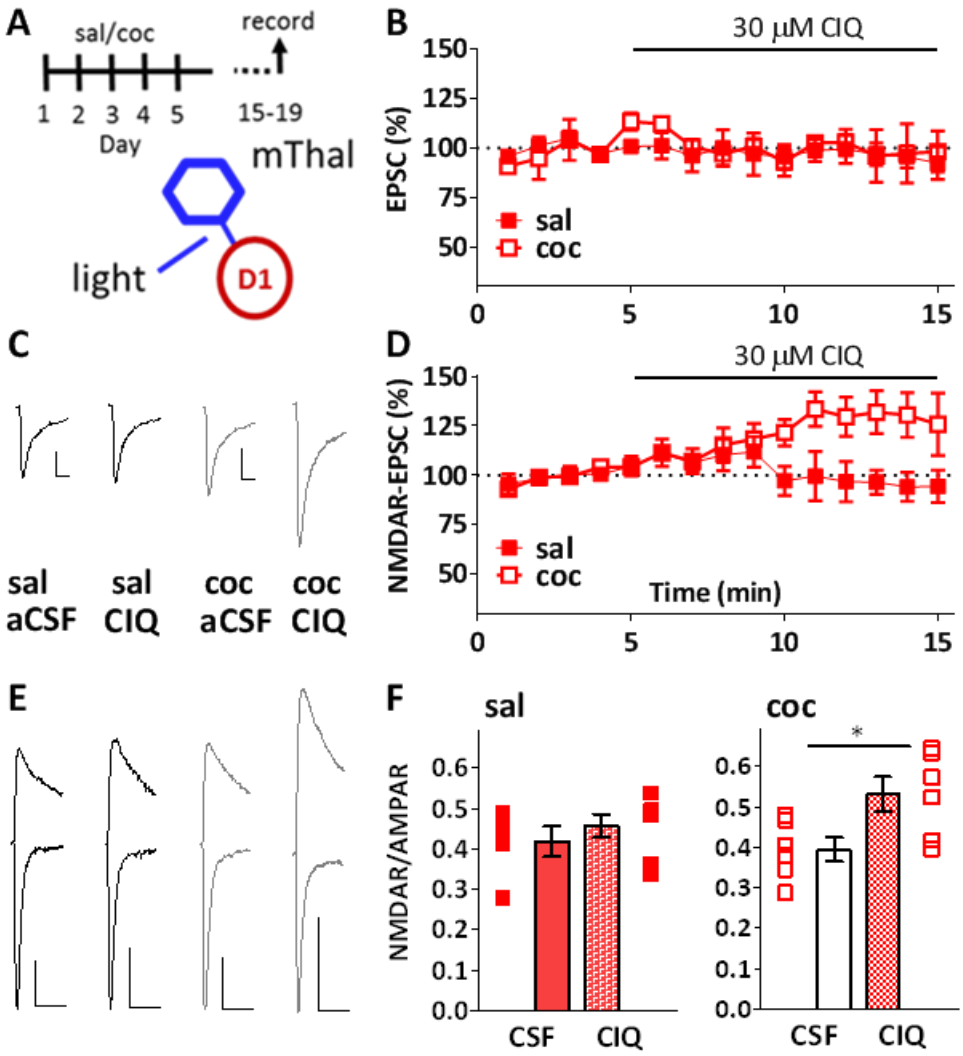


Figure 25. CIQ application reveals GluN2C/D function following cocaine experience. (A) Acute NAc core slices were prepared 2 weeks after mice were conditioned for 5 days with saline or cocaine. D1(+) neurons were targeted and glutamate was released at terminals from the mThal. (B) CIQ, a GluN2C/D positive allosteric modulator, did not alter EPSC amplitude after mice were treated with either saline (filled squares) or cocaine (open squares). (n/N = 4/3 saline; 3/3 cocaine). (C) Representative traces. Isolated NMDAR-EPSCs were elicited in low-Mg2+ aCSF and NBQX. Scale bars denote 100 pA and 50 ms. (D) Bath application of CIQ potentiated mThal-D1(+) NMDAR-EPSCs following cocaine experience. (n/N = 5/3 saline; 6/3 cocaine). (E) Representative traces. The NMDAR component was normalized to the AMPAR-EPSC in the presence of CIQ or aCSF. Scale bars denote 100 pA and 50 ms. (F) Left, CIQ did not affect mThal-D1(+) NMDAR/AMPAR in control slices. (n/N = 5/3 CSF; 7/3 CIQ). Right, CIQ enhanced NMDAR/AMPAR at mThal-D1(+) NAc core synapses following in vivo cocaine experience ($p < 0.05$). (n/N = 6/3 CSF; 6/3 CIQ).

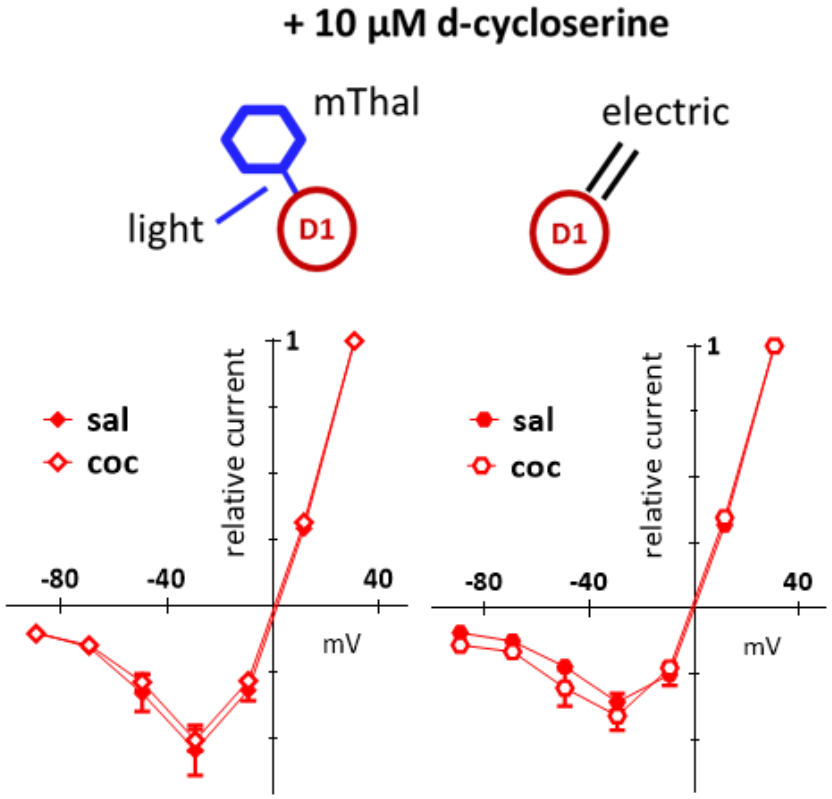


Figure 26. Inclusion of d-cycloserine in aCSF abrogates cocaine-induced difference in mThal-D1(+) NMDAR function. The NMDAR current-voltage relationship was assessed by eliciting isolated NMDAR-EPSCs at 20 mV steps and normalizing to the maximum outward current for each cell. D-cycloserine (10 µM) was added to the aSCF. Left, optically-evoked mThal-D1(+) NMDAR current-voltage plot. Right, electrically-evoked non-specific D1(+) NMDAR current-voltage plot. (n = 5-7 / N = 3 per group).

3.8 Cocaine Experience Unmasks NMDAR-dependent LTD at mThal-D1(+) NAc Core Synapses

Synaptic plasticity is commonly accepted as a substrate for learning and memory. Metaplasticity is an adaptation that promotes greater dynamic change in synaptic strength and connectivity. After determining that cocaine alters the stoichiometry of mThal-D1(+) NMDARs, we sought to test their function by analyzing synaptically-evoked NMDAR-dependent long-term plasticity (Brebner et al., 2005; Ma et al., 2014; Pascoli et al., 2012). In slices from mThal-ChR2 mice, 1

Hz 470 nm light stimulation for 10 minutes (low frequency stimulation; LFS) did not decrease the EPSC amplitude in D1(+) NAc core MSNs (sal: 94.0 ± 7.6%, Figures 27A-27D, page 105). However, LFS induced LTD of mThal-D1(+) EPSCs in slices from cocaine-conditioned mice (coc: 68.1 ± 6.2%, $p < 0.05$ vs. sal). Bath application of D-AP5 during LFS blocked LTD in D1(+) MSNs from cocaine-treated animals (92.6 ± 10.7%, Figure 28, page 106), corroborating an NMDAR-dependent mechanism of LTD. Since we observed that cocaine exposure induced changes in NMDAR function at a moderately depolarized holding potential, we paired the LFS with depolarization and expected to observe enhanced NMDAR function. Indeed paired LFS induced greater LTD at mThal-D1(+) synapses from cocaine-conditioned animals (sal: 83.9 ± 9.1% vs coc: 54.2 ± 7.5%, $p < 0.05$, Figures 27E-G). These data provide further evidence that cocaine experience alters NMDAR function at mThal-D1(+) NAc core synapses and identify a circuit-based substrate underlying pathophysiological learning and memory processes.

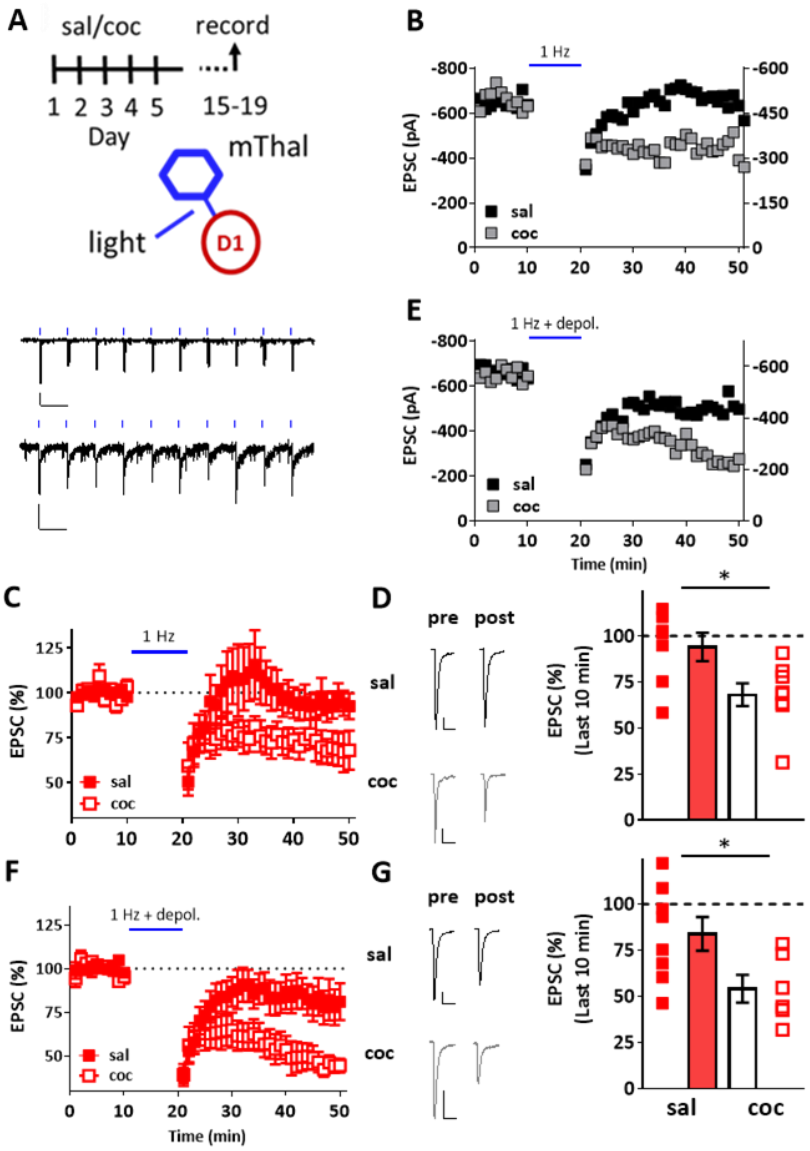


Figure 27. Cocaine exposure unmasks NMDAR-dependent synaptic plasticity in NAc core at mThal synapses on D1(+) neurons. (A) Top, schematic of behavioral conditioning and circuit-specific approach. Bottom, representative traces displaying low frequency stimulation (LFS: 1 Hz) of mThal-specific EPSCs at resting and depolarized holding potentials. (B) Representative LTD experiments following 10 minutes mThal-specific LFS. LFS exerted no effect on mThal-D1(+) EPSC amplitude in control cells (black) but induced LTD in D1(+) MSNs from cocaine-conditioned mice (grey). (C) Summary time course of NMDAR-LTD. (n/N = 7/7 saline; 8/7 cocaine). (D) Left, representative traces from the baseline (pre) and final 10 minutes of recording (post). Right, average EPSC amplitude during last 10 minutes. Cocaine exposure permits NMDAR-LTD ($p < 0.05$). (E) Representative LTD experiments. mThal-specific LFS was paired with depolarization. Paired LFS generated modest LTD in control D1(+) MSNs but greater LTD in cells from cocaine-conditioned animals. (n/N = 8/6 saline; 6/5 cocaine). (F) Time course of paired NMDAR-LTD. (G) Left, representative traces from the baseline and end of LTD recording. Right, average EPSC amplitude during last 10 minutes. Cocaine treatment enhanced LTD induction ($p < 0.05$).

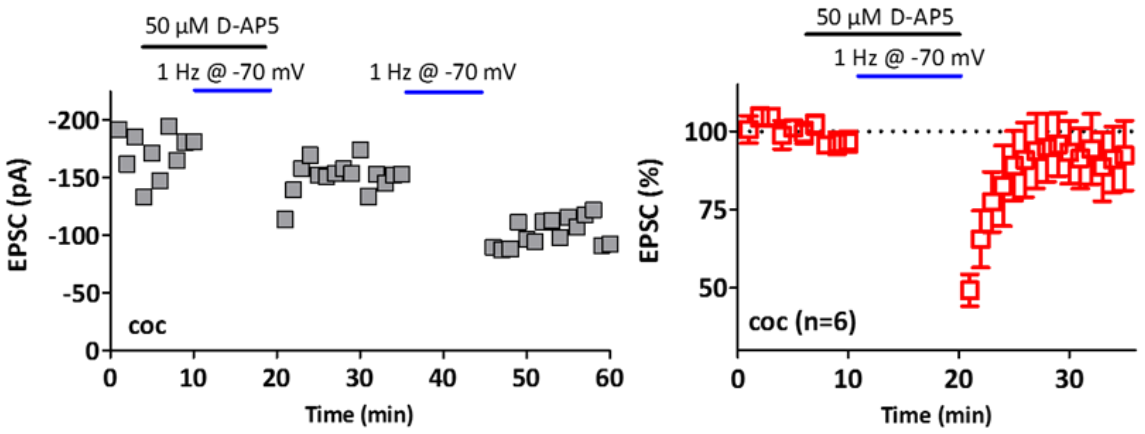


Figure 28. mThal-NAc LFS generates LTD via activation of NMDARs. Recordings were made from D1(+) NAc core MSNs in slices prepared from cocaine-conditioned animals. An NMDAR antagonist (D-AP5, 50 µM) was applied during the LTD induction period and prevented the protocol from decreasing the amplitude of the evoked mThal-D1(+) response. (n/N = 6/4).

4. Discussion

In this report we investigated AMPAR and NMDAR function across several NAc synapses, and further demonstrated that cocaine experience exerts disparate outcomes on these circuits. We highlighted changes in excitatory synaptic transmission from the mThal to the NAc core, as this overlooked projection could yield important insight into the integration of sensorimotor information towards the execution of motivated behaviors. As reported by MacAskill et al.(MacAskill et al., 2012), AMPAR function at mThal-D1(+) and mThal-D1(-) is similar to the synapses from other inputs under basal conditions. However, our results suggest differences in NMDAR function across inputs to the NAc core. We proceeded to assess how cocaine exposure and abstinence altered NAc core NMDAR function in a circuit-specific manner.

Consistent with previous reports (Dobi et al., 2011; Thomas et al., 2001), we found that cocaine exposure and withdrawal did not induce discernable

changes in electrically-evoked AMPAR or NMDAR function within the NAc core. Notably, these results differ from Jedynak et al. who noted that the same cocaine conditioning paradigm enhanced AMPAR function in the NAc core as assessed by electrically-evoked AMPAR/NMDAR and mEPSC amplitude. These discrepancies may be explained by technical differences, biology, or both. For one, Jedynak et al recorded peak isolated NMDAR currents at room temperature, whereas the values used in the present study reflect the late-phase dual component EPSC in a heated bath. As temperature is known to alter NMDAR decay kinetics (Losi et al., 2002), our methods were geared towards detecting differences in GluN2 subunit stoichiometry. Secondly, although asEPSCs and mEPSCs each reflect synaptic AMPAR function, nuanced differences between the measurements (exemplified by Thomas et al.) may explain the discrepancy in interpretations. From a biological standpoint, recordings made by Jedynak et al were performed in the medial NAc core (ML: 0.72-0.96 mm), whereas those from the present study were more lateral (ML: 1.0-1.25), and entirely dorsal to the anterior commissure. Relatively little attention has been paid to subregion-specific differences in NAc function (Al-Hasani et al., 2015; Britt et al., 2012), but these subtle anatomical/physiological gradations merit further study. Notwithstanding these minor differences, the present data emphasize the importance of considering input specificity when interrogating NAc physiology. When isolating mThal-D1(+) NAc core synapses, we observed that cocaine exposure and abstinence enhanced AMPAR function without affecting ratiometric measurements. By augmenting AMPAR function at mThal-D1(+) synapses, a previous cocaine history is expected to promote future

D1-MSN activation and associated reward-related behaviors (Lobo and Nestler, 2011), similar to inputs from the PFC, BLA, and vSub to the NAc shell (Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014, 2012).

In addition to increasing mThal-D1(+) AMPAR function, cocaine experience drove changes in NMDAR properties that enhanced their function. Specifically we found that following cocaine exposure, mThal-D1(+) NMDARs pass relatively more current at moderately hyperpolarized potentials and induce greater changes in downstream signaling. As the observed changes in NMDAR current-voltage relationship are consistent with the incorporation of GluN2C/D-containing NMDARs, we employed the GluN2C-preferring ligands, CIQ (Mullasseril et al., 2010; Ogden et al., 2014) and d-cycloserine (Dravid et al., 2010; Sheinin et al., 2001) to assess NMDAR subunit stoichiometry. CIQ selectively potentiated mThal-D1(+) isolated NMDAR EPSCs and NMDAR/AMPAR from cocaine-conditioned mice. Moreover, inclusion of d-cycloserine in the aCSF eliminated the cocaine-induced difference in NMDAR current-voltage relationship. In the context of alcohol abuse, PFC-NAc core GluN2C function has been demonstrated to promote compulsive drug-seeking (Seif et al., 2015, 2013). The present data provide the first evidence for the involvement of these NAc NMDAR receptors in psychostimulant exposure, and we speculate that the incorporation of GluN2C subunits at mThal-D1(+) synapses may be a permissive step towards the development of pathological drug-seeking behaviors. One interesting caveat is that d-cycloserine acts as an NMDAR partial antagonist when extracellular glycine concentrations are elevated (Hood et al., 1989; Krystal et al., 2011; Watson et al.,

1990), raising the possibility that NAc glycinergic transmission may be involved in the actions of cocaine (Achat-Mendes et al., 2012). Nonetheless, the convergent biophysical and pharmacological data presented here support a role for the GluN2C subunit in the pathophysiological effects of cocaine experience. However in cell lines (Paoletti et al., 2013; Siegler Retchless et al., 2012) and in NAc MSNs following alcohol intake (Seif et al., 2015, 2013), GluN2C subunits contribute to the EPSC at resting membrane potentials – a phenomenon we did not observe at mThal-D1(+) synapses. Instead our data resemble differences observed at thalamic inputs onto cortical principal neurons, which contributed greatly to the postsynaptic membrane potential and subsequent spiking (Hull et al., 2009). The unusual (but not unprecedented) difference in the current-voltage relationship suggests that cocaine experience enriches non-canonical GluN2C-containing receptors (i.e. heterotrimeric receptors) at mThal-D1(+) synapses. Testing this hypothesis requires tools not yet readily available (Paoletti and Neyton, 2007; Paoletti et al., 2013), but GluN2C-containing NMDARs represent an intriguing potential mediator of behaviors related to relapse.

At PFC-D1(+) synapses, we observed increased NMDAR/AMPAR in cocaine treated groups without a consistent change in the AMPAR-mediated component as measured by asEPSC amplitude. Further unlike the mThal-D1(+) synapses, PFC-D1(+) synapses did not exhibit any changes in NMDAR current-voltage relationship following cocaine experience. These findings suggest that cocaine experience increases NMDAR expression without altering subunit stoichiometry. Alternatively, enhancement of the NMDAR-EPSC may have been

generated by changes in the phosphorylation (Pascoli et al., 2011) and/or localization (Ghasemzadeh et al., 2009; Ortinski et al., 2013) of the receptors. A recent report demonstrated that abstinence from cocaine self-administration enhanced relapse-preventing NMDAR-dependent LTD at prelimbic PFC-NAc core synapses (Ma et al., 2014). Our data provide an explanation whereby an increase in NMDAR function provided the cell type-specific molecular mechanism required for the light-induced reversal of cocaine craving. We also found that cocaine experience had no detectable effect on AMPAR or NMDAR function at PFC-D1(-) synapses. These data support continuing translational efforts towards restoring pathophysiological changes induced on D1-expressing MSNs, and emphasize that interactions between NMDARs and D1-MSN-specific proteins provide a target for developing pharmacological therapies for addiction-related disorders.

The current body of literature supports the view that the majority of drug-induced adaptive changes in synaptic function occur in D1(+) and not D1(-) MSNs in the NAc (Lobo and Nestler, 2011). To the best of our knowledge, only one report has illustrated that psychostimulant exposure induced a change in AMPAR or NMDAR function at D1(-)/D2(+) NAc core MSNs (Bock et al., 2013). However, experiments following ablation (Durieux et al., 2009), transient inhibition (Bock et al., 2013), or activation (Bock et al., 2013; Lobo et al., 2010) of D2(+) NAc MSNs provide compelling evidence that these neurons do modulate the behavioral responses to psychostimulants. Our data indicate that excitatory synaptic transmission onto D1(-) MSNs is affected by cocaine experience, as we observed enhanced NMDAR/AMPAR at mThal-D1(-) synapses. This finding is

fundamentally different from our other observations of enhanced NMDAR function: the change in mThal-D1(-) NMDAR/AMPAR was accompanied by a decrease in CV-N/CV-A, suggestive of an increased proportion of silent synapses. Previous findings demonstrated that cocaine-generated silent synapses are important for persistent adaptations but exist themselves only temporarily (T. E. Brown et al., 2011; Huang et al., 2009b; Lee et al., 2013; Ma et al., 2014). Our data are the first to suggest that some populations of NAc silent synapses may persist beyond short-term abstinence. One intriguing hypothesis is that mThal-D1(-) silent synapses provide a substrate for subsequent strengthening of mThal-to-NAc core D1(-) MSN connectivity that might promote resilience to drug-seeking (Bock et al., 2013). In sum, the changes we described not only uncovered novel molecular processes and circuitry affected by cocaine experience, but provide mechanistic explanations for several impactful recent findings.

5. Coefficient of Variation Analysis

Table 2

Fig 13

		mThal	PFC	BLA	Two-way ANOVA
CV-N	**D1 (+)**	0.227 ± 0.025	0.141 ± 0.017	0.170 ± 0.019	***Input: F (2, 59) = 10.46, p < 0.0001*** Cell type: F = 0.14, Interaction: F = 0.09
	D1 (-)	0.243 ± 0.026	0.140 ± 0.016	0.174 ± 0.019	
CV-A	**D1 (+)**	0.206 ± 0.023	0.144 ± 0.010	0.168 ± 0.017	***Input: F (2, 59) = 4.55, p < 0.02*** Cell type: F = 0.71, Interaction: F = 1.03
	D1 (-)	0.226 ± 0.025	0.183 ± 0.025	0.150 ± 0.019	

Fig 14

		electric	
		saline	cocaine
D1 (+)	**CV-N**	0.204 ± 0.033	0.182 ± 0.013
	CV-A	0.169 ± 0.020	0.170 ± 0.020
D1 (-)	**CV-N**	0.258 ± 0.067	0.180 ± 0.036
	CV-A	***0.236 ± 0.030****	***0.166 ± 0.016****

Figs 15-18

		mThal		PFC	
		saline	cocaine	saline	cocaine
D1 (+)	**CV-N**	0.216 ± 0.017	0.186 ± 0.034	0.149 ± 0.015	0.133 ± 0.016
	CV-A	0.213 ± 0.016	0.197 ± 0.039	0.159 ± 0.022	0.133 ± 0.014
D1 (-)	**CV-N**	0.224 ± 0.029	0.172 ± 0.022	0.167 ± 0.025	0.178 ± 0.031
	CV-A	***0.198 ± 0.023****	***0.279 ± 0.028****	0.133 ± 0.017	0.141 ± 0.017

Table 2. Isolated CV analysis. CV of optically-evoked EPSCs in NAc core vary based upon input but not MSN type. Cocaine experience alters CV-A at D1(-) MSNs. BLA, basolateral amygdala; CV, coefficient of variation; CV-A, CV of AMPAR-EPSC; CV-N, CV of NMDAR component; mThal, midline nuclei of the thalamus; PFC, prefrontal cortex.

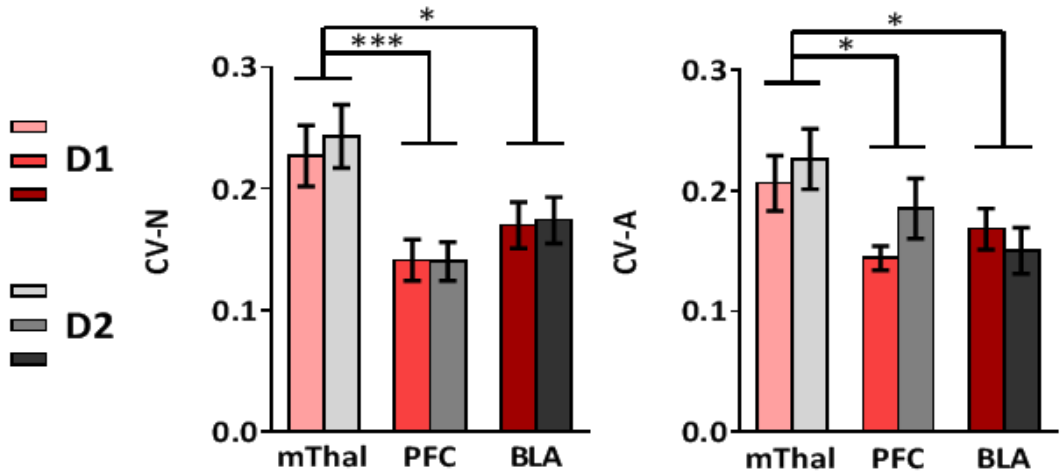


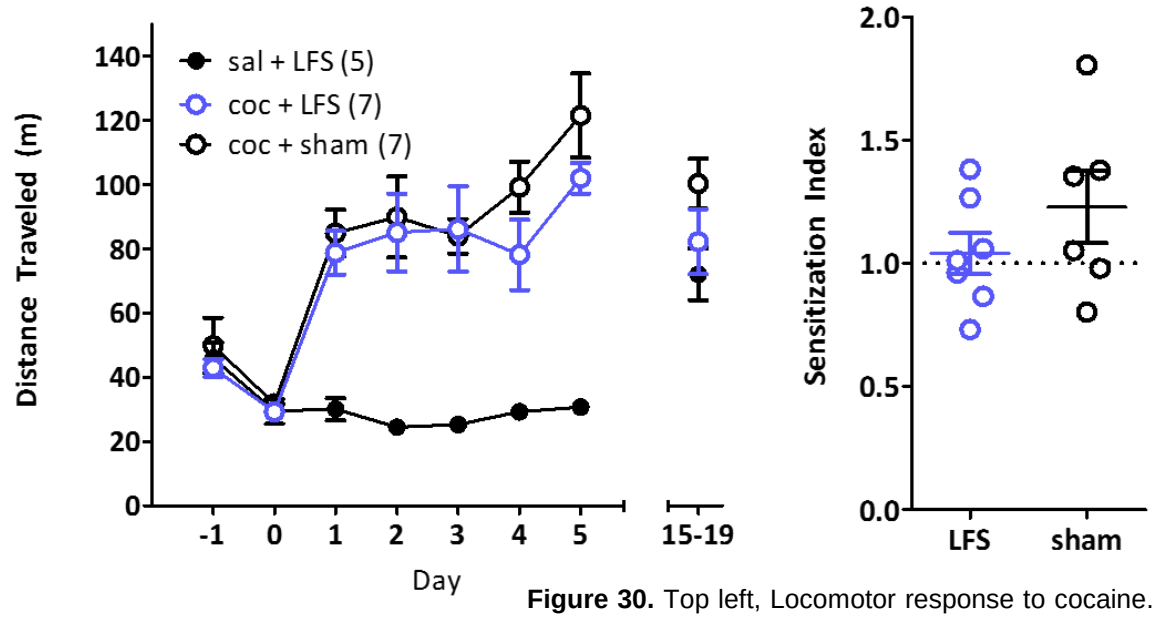
Figure 29. CV-NMDAR (CV-N) and AMPAR (CV-A) components of the circuit-specific EPSCs in NAc core. For both CV-N and CV-A, a two-way ANOVA revealed a main effect of input but the targeted cell type or an interaction. Inputs from the mThal displayed a greater CV-N and CV-A than inputs from the PFC and BLA (*: $p < 0.05$, ***: $p < 0.001$, post-tests). As depicted in Figure 1, the ratio of CV-N/CV-A was not different than 1 for any circuit under basal conditions. (n = 10-13 / N = 5-10 per group).

Across isolated inputs to the NAc core, we observed significant differences in AMPAR-CV and in NMDAR-CV, irrespective of MSN type or an interaction (Figure 29). According to quantal theory of neurotransmission (Kerchner and Nicoll, 2008), CV is inversely proportional to *n* (the number of synapses recruited) and *p* (presynaptic release probability), or collectively as *n*p* (quantal content). These data may suggest that mThal inputs display low quantal content relative to the PFC and BLA, however several caveats and confounds impair a clean interpretation. For one, these data cannot resolve whether differences may be due to *n*, *p*, or both. We did not measure the percentage of neurons expressing ChR2 in the mThal/PFC/BLA or the density of fibers reaching the NAc, so differences in expression fidelity could underlie variability in CV. Although we did not make rigorous evaluations, we consistently observed that mThal-NAc ChR2-EYFP

expression appeared brighter than that of NAc terminals from the PFC or BLA. Lower light intensities were also required to titer mThal-specific stimulation to yield EPSCs with small amplitudes (100 - 600 pA). Controlling stimulation parameters in this way is important for the interpretation of synaptic AMPAR and NMDAR properties. However we may have artificially decreased measurable quantal content relative to the other inputs, consistent with the greater CV we obtained from mThal inputs. With the exception of cocaine-conditioned mThal-D1(-) synapses, no population displayed a CV-NMDAR/CV-AMPAR different from 1. These data are consistent with the prevailing hypothesis that the adult NAc harbors minimal silent synapses under basal conditions (Lee and Dong, 2011). Examining CV-AMPAR and CV-NMDAR separately affirmed those findings. The isolated analyses also determined that electrically-evoked quantal content of AMPAR-containing synapses may actually increase on D1(-) MSNs following cocaine experience, however caution must be exercised when interpreting these data (*: $p < 0.05$, t-test).

6. *In vivo* optogenetics

We aimed to test a causal link between mThal-specific potentiation and locomotor sensitization (see Pascoli et al., 2012), by applying mThal-NAc NMDAR-dependent LTD (as characterized in Figures 27-28, pages 105-10) in vivo. Mice were injected with AAV-ChR2 or AAV-GFP in the mThal and conditioned with 5 days of cocaine or saline identically as those for electrophysiology. Midway through the abstinence period, a second surgery was performed to implant



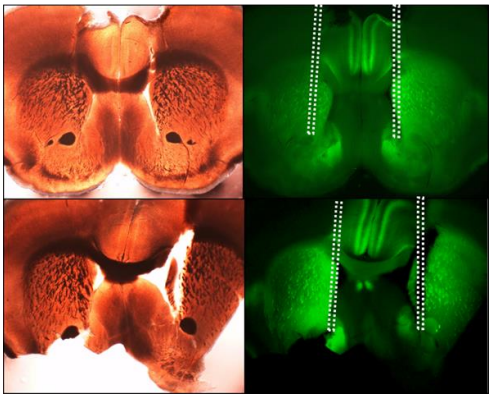


Figure 30. Top left, Locomotor response to cocaine. Prior to the challenge dose of cocaine, mice were administered 5 injections of cocaine and sham light treatment (coc + sham, black circles), 5 injections of cocaine and mThal-NAc 1 Hz stimulation (coc + LFS, blue circles), or 5 injections of saline and mThal-NAc 1 Hz stimulation (sal + LFS, filled black circles). Top right, Sensitization index (Locomotor activity of challenge injection normalized to Day 1) computed for groups that received prior cocaine exposure. Lower left, representative images of brain slices from two experimental mThal-NAc ChR2-EYFP mice with overlaid placement of fiberoptic cannulae.

chronically indwelling fiberoptic cannulae in the NAc core (relative to bregma, ML: ± 1.0, AP: 1.3, DV -4.0). On day 14, we applied 600 4-ms pulses at 1 Hz through the fiberoptic cannulae in the home cage. After the procedure and 30 minutes rest, mice were re-exposed to a challenge dose of cocaine and their locomotor activity was monitored, but no effect of the light treatment was observed (Figure 30). An unfortunate caveat to this and many in vivo optogenetic approaches is that a negative result is difficult to interpret. ChR2-EYFP expression in NAc axon terminals as well as cannulae placement were confirmed after all included experiments.

CHAPTER VI

DISCUSSION

1. Summary

The rewarding and reinforcing properties of cocaine and other drugs of abuse are largely mediated by activity within the NAc, a key component of the mesolimbic dopamine system. Excitation of neurons in the NAc has been described as the final common pathway for natural rewards and drugs of abuse (Joffe et al., 2014; Kalivas et al., 2005). Additionally, persistent drug- and withdrawal-induced synaptic changes in the NAc are thought to underlie the generation of maladaptive behaviors observed in addiction (Grueter et al., 2012). Synaptic plasticity involving NMDARs is being recognized as an important process in the development of addiction-like behaviors, but much remains to be learned about the specific circuitry involved. The work described in this dissertation adds to the literature by providing the first thorough account of drug-related changes to the mThal-NAc pathway as well as evidence for the contribution of D1(+) GluN2C-containing NMDARs to relapse-related behaviors.

1.1 D1 MSNs

To examine synapses from specific NAc core inputs, we recorded light-evoked EPSCs from acute brain slices following viral-mediated expression of ChR2 in the mThal, PFC, or BLA. To identify NAc MSN subtypes we utilized mice

expressing tdTomato driven by the D1R promoter, and we recorded AMPAR and NMDAR properties to evaluate synaptic adaptations induced by cocaine experience, a 5-day cocaine exposure followed by 2-weeks of abstinence. Cocaine experience induced contrasting changes in AMPAR and NMDAR properties at mThal-D1(+), mThal-D1(-), and PFC-D1(+) synapses, while PFC-D1(-) iGluR properties were unaffected. At mThal-D1(+) synapses, we demonstrated that cocaine alters the NMDAR current-voltage relationship and enhances NMDAR-dependent synaptic plasticity, consistent with an upregulation of GluN2C-containing NMDARs.

We uncovered that cocaine exposure induced long-term changes in NMDAR function on D1(+) NAc core MSNs at two distinct inputs. Interestingly these changes were qualitatively different. For one, enhanced NMDAR function at mThal-D1 synapses was accompanied by an increase in quantal size, whereas AMPAR function in the PFC-D1 circuit appeared unaffected. Secondly cocaine exposure altered the NMDAR current-voltage relationship at mThal-D1 synapses but not at PFC-D1, and further pharmacological experiments implicated GluN2C-containing NMDARs in this phenomenon. To assess whether NMDARs on D1R-expressing MSNs contribute to reward-related behaviors, we generated cell type-specific deletions by targeting the obligatory GluN1 subunit. Despite expressing typical cocaine reward learning, D1-GluN1$^{-/-}$ mice displayed a pronounced impairment of reinstatement, which suggests that these specific NMDARs may underlie susceptibility to relapse following cocaine exposure and abstinence.

Certainly, further experiments are required to ascertain causative relationships. For one, based on these experiments alone it is unclear that D1-NMDARs in the NAc core underlie reinstatement to cocaine seeking. Several complimentary approaches could be taken to evaluate this concern. For one site-directed viral-mediated manipulations of NMDAR function would provide enhanced spatial and temporal resolution. Rescuing reinstatement of cocaine CPP in D1-GluN1$^{-/-}$ mice by viral delivery of Cre-dependent *Grin1* to the NAc would address two concerns inherent to the genetic deletion: poor regional specificity and potential gross developmental abnormalities. On the other hand, a Cre-dependent viral-mediated knock-down would circumvent both of these concerns, but may not provide the sufficient efficiency required to observe behavioral effects. RNA interference directed at the GluN2C subunit (Seif et al., 2013) could yield valuable mechanistic insight potentially corroborating the observed electrophysiological changes.

A second approach would utilize in vivo optogenetics. We characterized that cocaine exposure unmasks NMDAR-dependent LTD at mThal-D1(+) synapses. Ma et al., 2014 described a similar phenomenon at inputs from the PFC and went on to demonstrate that its application in vivo altered incubation for cocaine-seeking. Applying such protocols in the NAc core prior to reinstatement for CPP would test that these specific NMDAR-dependent modifications are required for pathophysiological behavior, and we could control for cell type-specificity by also performing the experiment in GluN1$^{-/-}$ mice. We could combine this approach with

subunit-specific pharmacology (Acker et al., 2011; Costa et al., 2009; Lozovaya et al., 2014) to pinpoint the involvement of GluN2C-containing NMDARs

Despite these potential follow up experiments, an unresolved question would remain. Do NMDAR-dependent modifications in the NAc ultimately promote relapse or is NMDAR function itself required for the expression of maladaptive relapse-like behavior? Put another way, what is the final common pathway of the final common pathway: AMPAR function or NMDAR function? In reality, this is likely a false dichotomy. Several recent experiments have used in vivo LTD protocols to demonstrate that depotentiation of NAc excitatory synapses prevents relapse-like behaviors (Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014, 2012). The conclusions drawn from these experiments have been that abnormal NAc AMPAR function subserves reinstatement, but the impact of these LFS protocols on NMDAR function (and mGluR function) has been ignored. Conventional wisdom dictates that AMPAR function is more fluid, but the surface expression and localization of NMDARs is dynamically regulated nonetheless (Papouin et al., 2012). With the present research and other recent accounts (Seif et al., 2015, 2013), we hope the hypothesis that NAc NMDAR function promotes addiction-like behavior is more thoroughly tested.

Some of the most meaningful data from this work may be that cocaine exposure increases the function of mThal-D1-NMDARs at resting potentials. Even when the MSN was clamped near a typical MSN resting membrane potential, we could generate NMDAR-dependent LTD at mThal-D1(+) synapses following cocaine conditioning. Pharmacological experiments confirmed upregulation of

GluN2C-containing NMDARs, however the change in the current-voltage relationship was admittedly unusual. In cell lines, GluN1/GluN2C homodimers typically pass more current at all hyperpolarized potentials, including those near the resting membrane potential (Paoletti et al., 2013). In contrast to canonical data, the change in the current-voltage relationship we observed occurred specifically at moderately hyperpolarized potentials (e.g. -30 mV). There are several possible hypotheses that could explain the unique change we observed. For one, protein-protein interactions and/or modifications that affect NMDAR current-voltage relationship (Chen and Huang, 1992; Lu et al., 1999) could be affected by a prior cocaine experience. Second, local concentrations of Mg^{2+} could be altered following cocaine. And finally, although the tools to best test this hypothesis are lacking (Paoletti and Neyton, 2007), triheteromeric NMDARs with uncharacterized biophysical properties could be assembling in the NAc core. In fact, we have obtained preliminary evidence that points towards the GluN2B subunit as a key regulator of voltage dependence in the NAc core.

1.1.1 Verification of Cell Type-specific GluN2B Deletions

We generated cell type-specific deletions of the GluN2B subunit by crossing mice expressing $Grin2B^{lox/lox}$ with D1- and A2A-BAC-Cre mice, in an analogous strategy as to the $GluN1^{-/-}$ lines. However for the electrophysiological verification of this line, we also crossed these mice to Ai9 mice, which contain a Cre-inducible tdTomato construct under control of the ubiquitously expressed Rosa26 promotor. Therefore for the verification of $Glun2B^{-/-}$, MSNs in the NAc core were A2A(+) and

A2A(-) MSNs were assumed equivalent to D1(-) and D1(+) MSNs respectively. GluN2B-containing NMDARs exhibit longer decay kinetics than GluN2A counterparts, therefore we isolated NMDAR-EPSCs from targeted MSNs to evaluate the specificity of the genetic deletions. Indeed NMDAR-EPSCs from both D1-Glun2B$^{-/-}$ and A2A-Glun2B$^{-/-}$ MSNs exhibited faster decay times than WT control cells (Figure 31).

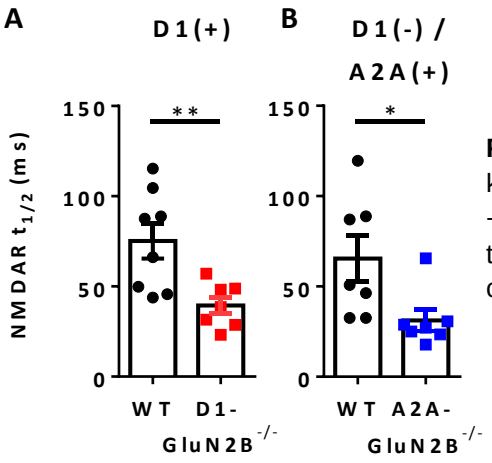


Figure 31. GluN2B deletions reduce NMDAR decay kinetics. Isolated NMDAR currents were evoked at +40 mV. Genetic deletion of GluN2B increased the time to decay 50% in both D1 (A) and A2A (B) NAc core MSNs. (*: $p < 0.05$, **: $p < 0.01$, t-tests).

We also analyzed the I-V relationship of NMDAR EPSCs. In cell lines GluN1/GluN2B homodimers display similar I-V relationships to GluN1/GluN2A receptors. Surprisingly we observed that genetic deletion of GluN2B from NAc core MSNs altered the current-voltage relationship of NMDAR EPSCs at both D1 and A2A cell types (Figure 32, page 122). Subsequent post tests revealed differences in moderately hyperpolarized resting potentials, similar to the change we described following cocaine exposure at mThal-D1 synapses. Although rigorous examination of heterotrimeric receptors in native systems has proven elusive, the collective findings in this dissertation suggest that GluN1/GluN2B/GluN2C NMDARs may

assemble in NAc core MSNs. Further biochemical and functional experiments are needed to test this hypothesis.

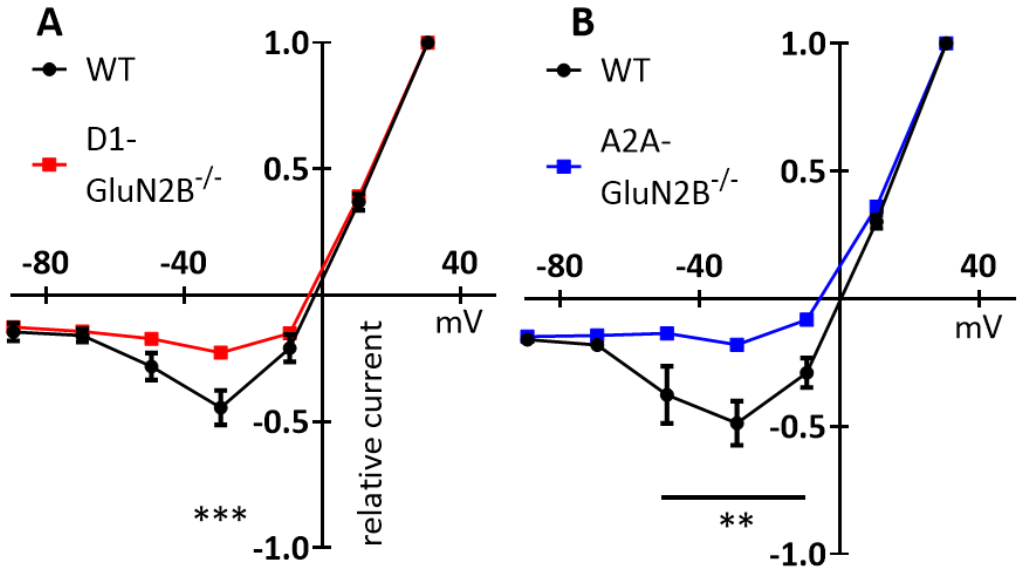


Figure 32. GluN2B genetic deletions modulate NMDAR current-voltage relationships. Current-voltage relationship of NMDAR currents in D1 (a) or A2A (b) NAc core MSNs. All currents are normalized to the peak current elicited at +40 mv. Control MSNs display characteristic inward rectification at hyperpolarized potentials. Knockout of GluN2B from either D1 or A2A MSNs further decreases the current passed at hyperpolarized potentials (**: $p < 0.01$, ***: $p < 0.001$, Bonferonni post tests). n = 6-7/group.

1.1.2 Assessment of Cocaine Reward Learning in GluN2B$^{-/-}$ Mice

To examine how MSN GluN2B signaling modulates the rewarding properties of cocaine, we performed cocaine CPP. We used a modified one-pairing procedure to assess submaximal reward learning. Following the pretest on day 1, mice were conditioned on day 2 with one pairing of cocaine (20 mg/kg, i.p.) in the less-preferred context. On day 3 mice were given free access to both sides of the chamber, and the increase in time spent was taken as an index of cocaine reward. We observed a modest degree of cocaine CPP in WT mice (127 ± 50 s, $p < 0.05$,

Figure 33A) and no differences were observed across genotypes (F(2, 31) = 0.1282, n.s.). We then followed up with 3 additional pairings of cocaine and vehicle, which generates maximal CPP in our laboratory. Again, and consistent with the data we obtained with GluN1$^{-/-}$ mice, all groups of mice exhibited CPP to a similar degree (F(2, 34) = 0.4594, n.s., Figure 33B), indicating that cell type-specific GluN2B function is not required for the acquisition of cocaine CPP.

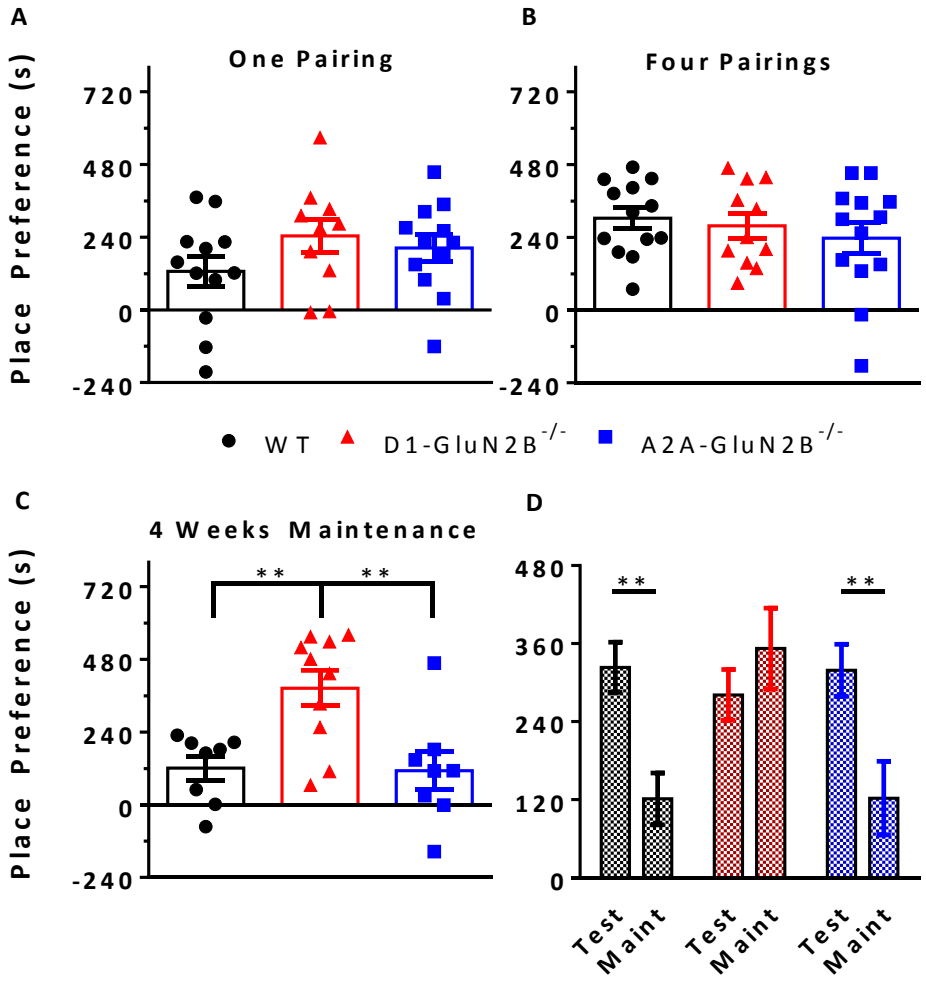


Figure 33. Expression and maintenance of cocaine CPP in GluN2B$^{-/-}$. (A) Submaximal cocaine CPP following one pairing of 20 mg/kg cocaine. (B) Saturated CPP following 4 total cocaine pairings. (C) Maintenance of cocaine CPP after 4 weeks. D1-GluN2B$^{-/-}$ mice exhibit enhanced retention of cocaine CPP relative to WT and A2A-GluN2B$^{-/-}$ (**: $p < 0.01$, Bonferonni post tests). (D) CPP at maintenance time point relative to expression test. WT and A2A -GluN2B$^{-/-}$ mice lost cocaine CPP 4 weeks after 4 weeks elapsed whereas D1-GluN2B$^{-/-}$ mice retained memory (**: $p < 0.01$, Bonferonni post tests). N = 8-10/group.

NAc GluN2B-expressing silent synapses have been implicated in the development of long-term behavioral effects of cocaine (i.e. incubation of cocaine-seeking). Therefore we sought to measure the maintenance of reward-related memory as assessed by retention of CPP 4 weeks later. Interestingly we observed a main effect of genotype on the maintenance of cocaine CPP at 4 weeks ($F(2, 23) = 8.186$, $p < 0.01$, Figure 33C). Follow-up comparisons revealed that D1-Glun2B$^{-/-}$ mice exhibited enhanced CPP at 4 weeks relative to WT controls and A2A-Glun2B$^{-/-}$ (D1: 384 ± 59 s vs WT: 121 ± 40 s, $p < 0.01$; A2A: 112 ± 63 s, $p < 0.01$). Differences at an intermediate 2-week time point were not observed (data not shown). We proceeded to compare the 4 week time point to the original test session in order to evaluate whether D1-Glun2B$^{-/-}$ mice exhibit "incubation" of cocaine reward or resistance to memory weakening (Figure 33D). At 4 weeks following the test session, WT mice displayed a reduction in cocaine CPP (Maint: 121 ± 40 s vs Test: 303 ± 34 s, $p < 0.01$). In contrast, 4 weeks elapsed time did not affect the CPP exhibited by D1-GluN1$^{-/-}$ mice (Maint: 384 ± 59 s vs Test: 278 ± 41 s, n.s.). Similar to WT mice, A2A -Glun2B$^{-/-}$ mice displayed a loss of cocaine CPP over the 4 week period (Maint: 112 ± 63 s vs Test: 237 ± 51 s, $p < 0.01$). Taken together these data suggest that loss of GluN2B function specifically from D1-expressing cells enhances long-term maintenance of cocaine reward memory. This phenomenon could arise from impairment of a memory weakening machinery or the initial formation of a more persistent memory.

The finding that D1-GluN2B function promotes the weakening of reward memory raises an interesting question about the cocaine-conditioned changes we

observed in NMDAR biophysical properties at mThal-D1(+) NAc core synapses. Does enhanced NMDAR function drive “forgetting” or extinction learning of reward memories? Perhaps instead of contributing to the likelihood of relapse, the enhanced expression of GluN2C-containing NMDARs serves as a feedback mechanism. In that case, the unmasked LTD we observed may be analogous to a process that occurs endogenously over the following weeks or months to attenuate long-term effects of cocaine exposure. Although much less is known about GluN2C-containing NMDARs, GluN2B subunits in other brain regions have been implicated in behavioral flexibility (Brigman et al., 2013; Dalton et al., 2011) and the extinction of fear (Dalton et al., 2012; Sotres-Bayon et al., 2009) and drug (Otis et al., 2014) memories. Together, these data suggest the possibility that NMDARs containing GluN2B (and potentially GluN2C/D) assemble after highly salient experiences and act to weaken, extinguish, or even reverse those memories.

The incubation of cocaine seeking in operant tasks has been characterized as requiring the presence of GluA2-lacking AMPARs (Conrad et al., 2008; Lee et al., 2013; Loweth et al., 2014, 2013; Ma et al., 2014). We hypothesize that GluA2-lacking AMPARs assemble in NAc MSNs following loss of GluN2B function, and thus promote the maintenance or “incubation” of the cocaine CPP memory. Their formation would be expected to increase the persistence of the cocaine-related memory duration and could explain the enhanced maintenance of the memory at 4 weeks. Alternatively, a more durable reward memory could have been formed due to enhanced connectivity, consistent with the finding that GluN2B knockdown increased the number of synaptic contacts In developing CA1 pyramidal neurons

(in contrast to GluN2A knockdown which modulated quantal size) (Gray et al., 2011). NAc NMDAR activity is generally considered to be a key player in the acquisition of reward-related memories and behaviors, but clearly, more experiments need to be performed to address whether NAc NMDARs act to enhance extinction or drive reinstatement (or both!). Cell type-specific deletions of NMDARs represent an attractive tool set to carefully craft studies that examine the electrophysiological, biochemical, and morphological changes induced in NAc MSNs by rewarding experiences.

1.2 A2A MSNs

Consistent with the literature (Bock et al., 2013; Dobi et al., 2011; Pascoli et al., 2014, 2012), we observed that cocaine exposure induced modest electrophysiological effects on D1(-) MSNs. For one, no differences between the cocaine and control groups were identified following local electrical or PFC-specific stimulation. However when eliciting glutamate release from mThal afferents, we observed that cocaine exposure induced changes most consistent with the incorporation of silent synapses. Silent synapses express functional NMDARs in the absence of AMPAR currents (Malenka and Nicoll, 1997) and are likely pivotal substrates for future plasticity due to their abilities to undergo major changes in synaptic strength (Kerchner and Nicoll, 2008). Despite the finding that cocaine exposure upregulates NMDAR function at mThal-D1(-) synapses, neither A2A-specific genetic deletion of GluN1 nor GluN2B affected cocaine reward learning as assessed by CPP. We speculate that these silent synapses may mature following

longer durations of cocaine exposure, drug self-administration, or co-administration with an aversive stimulus. For example, a recent report demonstrated that neither passive cocaine administration nor self-administration of a moderate-high dose of cocaine induced changes in AMPAR function on A2A NAc MSNs (Terrier et al., 2015). However long access self-administration of a very high dose (1.5 mg/kg/inf), which likely has some aversive properties, did drive increases in GluA2-lacking AMPAR function on A2A MSNs. As suggested by other studies (Lee et al., 2013; Ma et al., 2014), GluN2B-enriched silent synapses may exist as a necessary intermediate. Therefore the silent synapses generated at mThal-D1(-) NAc synapses could provide an essential transition step between initial cocaine exposure and addiction-like behaviors such as punishment-resistant drug seeking

In contrast to reward-related behaviors, A2A-NMDARs do modulate behavioral despair. Specifically A2A-GluN1$^{-/-}$ mice displayed less immobility in the TST and FST relative to WT controls. These results are consistent with a report from Schwartz et al., 2014 who demonstrated that the expression of anhedonia following chronic pain requires NMDAR-mediated plasticity on A2A MSNs. The present data provide evidence that even short-term stress-related behavioral adaptations may necessarily proceed through A2A-NMDARs. Functional experiments must be performed in order to assess the basal state of A2A-GluN1$^{-/-}$ mice and how NMDAR and AMPAR function in the NAc is affected by acute stressors. The mThal nuclei are highly interconnected with stress-related structures such as the BNST and BLA (Li and Kirouac, 2012; Penzo et al., 2015;

Phillipson, 1988; Vertes and Hoover, 2008), and represent an excellent candidate source of glutamate promoting behavioral despair and anhedonia. Future experiments should be directed at the potential role of the mThal-NAc pathway in responses to stress and the expression of anhedonia.

The present data provide further evidence for divergent behavioral influences exerted by D1 and A2A MSNs. A natural extension of this work is to examine how MSN efferent projections, and related inhibitory synaptic plasticity, regulate long-term behavioral adaptations related to stress and reward. Of particular interest is the VP, which receives input from both D1 and A2A MSN types (Kupchik et al., 2015). In turn, the VP sends projections to the mediodorsal thalamus (MD) (Donnell et al., 1997; Groenewegen, 1988; Mogenson et al., 1987). The MD then sends projections to the lateral NAc and ventral caudate-putamen (Berendse and Groenewegen, 1990; Cheatwood et al., 2003), producing a lateralizing ascending spiral analogous to the serial reciprocal circuits that link the midbrain and striatum (Belin and Everitt, 2008; Haber et al., 2000). Despite increased awareness of VP regulation of affective states and reward behaviors, much remains to be learned about the circuit-level logic that integrates NAc projections to the VP.

In the dorsal and ventral striatum, MSNs have been classically dichotomized by protein expression and anatomy. Conventional wisdom dictated that D1-expressing, striatonigral MSNs project to the midbrain (“direct pathway”), while A2A/D2-expressing, striatopallidal MSNs project to pallidal regions (“indirect pathway”), and exert differential control over motor activity (Groenewegen, 2003;

Kreitzer and Malenka, 2008; Smith et al., 2013). However in recent years several studies have come to question the legitimacy of separating of “direct” and “indirect” pathway neurons, especially in the NAc (Cazorla et al., 2014a; Saunders et al., 2015). In particular Kupchik et al., 2015, demonstrated that the distinction does not apply well to NAc MSNs, in that half of VP neurons received input from D1 MSNs (almost all VP neurons received input from D2 MSNs). Moreover, VP neurons that project to the MD also received inhibitory projections from D1 MSNs. These data pose many questions about how VP neuron populations may vary based on NAc afferent, or targets, and how these different populations may be recruited by rewarding and/or aversive experiences. For example, Mahler et al. demonstrated that inhibition of rostral VP neurons (which receive projections from the NAc shell), but not caudal VP neurons (which receive projections from the NAc core), blocks the response to cocaine associated cues. We speculate that these drug cue-associated behaviors may necessarily proceed through a thalamo-striatal-pallidal loop, but this hypothesis and its mechanistic underpinnings remain to be tested.

2. Future Examinations of mThal Function

2.1 mThal-NAc Presynaptic Function

The work described in this dissertation is focused on postsynaptic changes induced by cocaine exposure, but changes to the presynaptic release machinery can play a major role in synaptic plasticity and learning and memory (Malenka and Bear, 2004). With that in mind these findings pose many questions about how glutamate release probability may be altered at mThal-NAc synapses by in vivo

cocaine exposure. Presynaptic release probability can be assessed by a variety of measures, each with their own set of caveats. These measurements include: (1) frequency of miniature EPSCs (mEPSCs), (2) paired-pulse ratios (PPR), and (3) multiple probability fluctuation analysis (MPFA). (Suska et al., 2013) demonstrated that both non-contingent and self-administered cocaine exposure enhances glutamate release probability in the NAc shell specifically from the PFC and not BLA. To date, no other thorough examinations of input-specific NAc glutamate release probability have been published in peer-reviewed journals. However Neumann et al presented that cocaine self-administration enhanced release probability at PVT-NAc shell synapses at the most recent Society for Neuroscience Annual Meeting (Neumann PA, Graziane N, Huang YH, Xu W, Sesack SR, Nestler EJ, Schluter OM, 2015).

To further probe drug-related changes in presynaptic release machinery, future studies should examine short- and long-term synaptic plasticity. Within the striatum several neurotransmitter receptors participate in presynaptic plasticity, including CB1R, Group II mGluRs, and opioid receptors. These receptors decrease presynaptic release probability through Gi/o-proteins, whose signaling acts to inhibit cAMP production, interfere with SNARE complexes, and modulate a host of potassium and calcium channels. As a first assessment of mechanisms that regulate mThal-NAc presynaptic release probability, we elected to study the function of CB1R.

2.1.1 CB1R

To assess canonical CB1R-mediated short-term plasticity, we examined DSE from targeted MSNs (Shonesy et al., 2013). DSE occurs when depolarization of the clamped postsynaptic neuron generates a retrograde feedback signal that temporarily dampens presynaptic glutamate release. While we did not confirm a CB1R mechanism underlying LTD at these synapses, DSE has never been demonstrated to occur through other signaling pathways. When selectively releasing glutamate from mThal terminals, we successfully elicited DSE on both D1(+) and D1(-) MSNs (Figure 34A, page 132). A two-way ANOVA revealed a main effect of time ($F(35, 175) = 7.429$, $p < 0.0001$), as well as a significant interaction between time and cell type ($F(35, 175) = 1.617$, $p < 0.05$), likely owing to differences in the initial magnitude of the short term plasticity. DSE can also be elicited at both NAc core MSN types following PFC-specific or local electrical stimulation (data not shown).

To test that in vivo cocaine experience modulates presynaptic short-term plasticity, we elicited mThal-specific DSE from MSNs prepared from mice with a cocaine history or saline controls. When recording from mThal-D1(+) NAc core synapses, saline control cells exhibited a similar degree of DSE to non-conditioned naïve controls. However prior cocaine treatment increased the magnitude of short-term depression induced by DSE (coc: 62.4 ± 4.4% vs. sal: 75.8 ± 2.7%, $p < 0.05$, Figure 34B). In contrast, DSE generated at mThal-D1(-) synapses was unaffected by prior cocaine history (coc: 60.9 ± 3.2% vs. sal: 56.2 ± 5.0%, n.s., Figure 34C). After 4 weeks abstinence, the short-term depression at mThal-D1(+) synapses

returns to control values (coc: 69.1 ± 4.1% vs. sal: 74.3 ± 2.8%, n.s., data not shown).

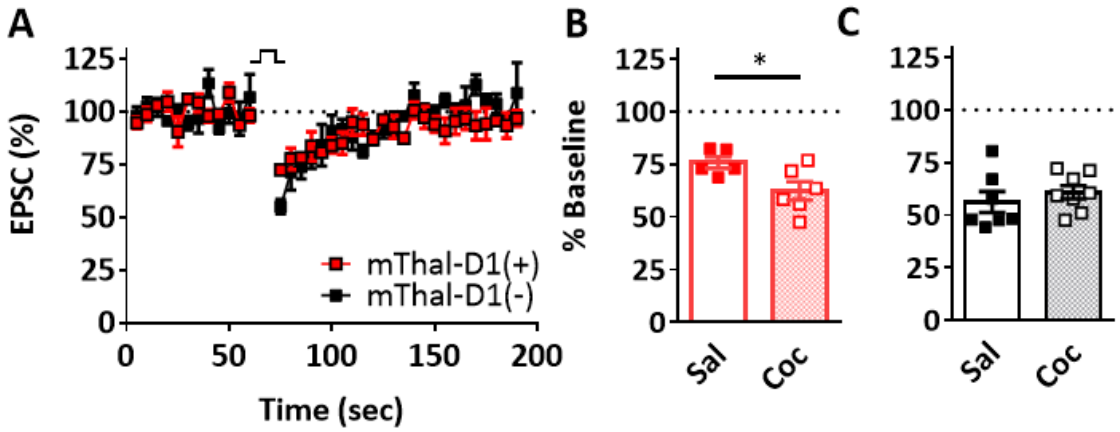


Figure 34. Cocaine exposure enhances mThal-D1(+) depolarization-induced suppression of excitation (DSE). (A) DSE can be elicited from mThal projections to both D1(+) and D1(-) MSNs in NAc core. (n = 4 D1(+); 3 D1(-)). (B) Abstinence from cocaine exposure enhances DSE at mThal-D1(+) synapses (*: $p < 0.05$). (n/N = 5/3 saline; 6/3 cocaine). (C) Summary mThal-D1(-) DSE from naïve controls, saline controls, and cocaine-conditioned mice. (n/N = 7/4 saline; 8/4 cocaine).

These data describe an additional means by which cocaine exposure perturbs normal function of mThal-D1(+) synapses. Enhanced DSE at mThal-D1(+) synapses is consistent with the increase in quantal size observed insofar as potentiated synapses are expected to permit greater relative short-term depressions. An alternative intriguing hypothesis is that tonic eCB or opioid production may normally occlude DSE at mThal-NAc synapses. Consistently, cocaine exposure has been shown to impair $mGlu_5$-mediated eCB production at these or nearby synapses (Fourgeaud et al., 2004; Grueter et al., 2010; Swanson et al., 2001; Szumlinski et al., 2004). If following cocaine exposure tonic $mGlu_5$-dependent eCB production is lost, but activity-dependent eCB production remains, we would expect to observe an enhancement of DSE. Future experiments will be

needed to assess the mechanism of this short-term plasticity and to contrast the molecular mediators with those in the $mGlu_5$-CB1R-TRPV1 pathway.

2.1.2 $mGlu_{2/3}$

Other regulators of glutamate release relevant to drug abuse behaviors and found in NAc terminals are the Group II mGluRs, $mGlu_2$ and $mGlu_3$. These targets have received significant attention in recent years (Johnson and Lovinger, 2015; Moussawi and Kalivas, 2010), and $mGlu_2$-selective positive allosteric modulators (PAMs) represent exciting potential clinical candidates (Salih et al., 2015). Two parallel recent studies have demonstrated than a selective $mGlu_2$ PAM inhibits drug-seeking behaviors in both rats (Caprioli et al., 2015) and squirrel monkeys (Justinova et al., 2015), but a better understanding of the relevant neurocircuitry is required to tailor treatments and mitigate off-target effects.

Inhibition of glutamate release by Group II mGluRs have been observed throughout the limbic system (Grueter et al., 2005; Liechti et al., 2007; Robbe et al., 2002). In the NAc, Group II mGluR-mediated depression of synaptic transmission occurs with a concomitant increase in PPR and specific reduction of mEPSC frequency, consistent with a presynaptic site of action (Manzoni et al., 1997). Perfusion of an $mGlu_{2/3}$ antagonist increases glutamate efflux in the NAc (Xi et al., 2002), suggestive of tonic a basal level of activation from tonic glutamate levels. Throughout the corticolimbic system, exposure to drugs of abuse has been shown to uncouple $mGlu_{2/3}$ from G-proteins (Liechti et al., 2007), likely through activator of G-protein signaling 3. Thus $mGlu_{2/3}$ receptors carefully buffer

glutamate release, and their impairment by drug exposure may be related to behavioral inflexibility observed in clinical populations.

The PVT nucleus expresses very high levels of Group II mGluRs, especially $mGlu_2$ (Ohishi et al., 1998, 1993; Petralia et al., 1996). Functionally, $mGlu_{2/3}$ activation has been shown to reduce excitability of PVT neurons through coupling to potassium channels, but their role at mThal-NAc terminals and in drug-related behaviors remains known. However we hypothesize that positive allosteric modulation of $mGlu_{2/3}$ attenuates neurotransmission at mThal-NAc core synapses and contributes to the observed anti-relapse behavioral effects. Furthermore, although the pharmacology used in historical studies was non-selective, newly developed compounds (i.e. $mGlu_3$ NAMs) permit the demarcation of Group II mGluR function (Walker et al., 2015; Wenthur et al., 2013) , and the testing of exciting hypotheses with translational relevance.

2.1.3 Opioid Receptors

As currently described, the opioid system consists of four distinct receptor-ligand systems (Mu, Delta, Kappa, and opioid receptor like-1), each of which have demonstrated action in the NAc (Al-Hasani and Bruchas, 2011). As the target of heroin and morphine, the Mu opioid receptor (MOR) is well known for its role in reward and reinforcement. One well-known dichotomy in striatal opioid signaling is that D1 MSNs express dynorphin (KOR agonist) whereas A2A/D2 MSNs express enkephalin (DOR agonist) (Le Moine et al., 1990; Lobo and Nestler, 2011). However the site of action of these peptides remains unclear, as the receptors are

expressed on multiple striatal cell types as well as on various axon terminals in the region. Within the dorsal striatum, recent research has demonstrated that Delta opioid receptor (DOR) mRNA expression colocalizes with *Drd2*, whereas MOR transcript is found in both MSN types, but to a greater degree in D1 MSNs (Banghart et al., 2015; Cui et al., 2014).

In humans MOR availability is greater in the thalamus than other MOR-expressing brain regions (Sprenger et al., 2005), and in the rat the PVT nucleus contains the highest levels of MOR immunoreactivity in the thalamus. The mThal is enriched with transcripts for MOR and KOR, but not DOR. Additionally, agonists of MOR, but not DOR, have been shown to directly hyperpolarize mThal neurons (Brunton and Charpak, 1998). Consistent with these early findings, a recent paper from the lab of David Lovinger (Atwood et al., 2014) demonstrated that in the dorsal striatum, MOR-LTD is expressed specifically at excitatory inputs from the thalamus but not from the motor cortex. In contrast, DOR-LTD was expressed at motor cortex terminals but not at projections from the thalamus. Furthermore thalamostriatal MOR-LTD was lost in the dorsal striatum following in vivo morphine exposure, and similar opioid signaling has been observed in the NAc (Hoffman and Lupica, 2001). These findings suggest that maladaptive opioid signaling at mThal-NAc terminals may underlie some of the behavioral effects of drugs of abuse. Although these findings raise many questions about opioid signaling at mThal-NAc synapses, this area of research remains largely unexplored.

One intriguing hypothesis is that the opioid systems may induce divergent behavioral effects in part through their actions on distinct elements of striatal

circuitry. For example recent work by Al-Hasani et al., 2015, demonstrated that, through KOR, stimulation of dynorphinergic NAc shell neurons can be either rewarding or aversive based on dorsal-ventral location. Afferents to the NAc are known to exhibit differential patterns of innervation along various anatomical gradients (Britt et al., 2012; Friedman et al., 2002; Phillipson and Griffiths, 1985). As KOR activation can inhibit neurons in the anterior PVT (Chen et al., 2015), one potential explanation is that the aversive actions of dynorphin are mediated through inhibition of PVT afferents to the ventral shell, but that KOR receptors in the dorsal shell promote reward through an alternative source of glutamate. Circuit-specific opioid signaling in the NAc remains a fascinating area of research, with implications for the regulation of complex behaviors and unresolved mechanisms of circuit-level and cellular signaling

2.2 mThal Neuron Function

In general, much remains to be learned about how the function of mThal neurons is altered following drug-related experiences in vivo. Little is known about the excitability of these neurons, and even less is known about the synaptic physiology of the inputs into this region. For simplicity, this discussion will be restricted to cover the PV, which has been the best characterized of the mThal nuclei. However future studies aimed at examining the PT and other mThal nuclei are certainly warranted.

2.2.1 Synaptic Physiology

Early anatomical studies showed that the PVT receives projections from hindbrain regions that are associated with function of the autonomous nervous system (Chen and Su, 1990; Cornwall and Phillipson, 1988). The PVT receives major input from the PFC and insular cortices, as well as relatively minor input from the BNST and amygdalae (Groenewegen, 1988; Li and Kirouac, 2012; Vertes et al., 2015). Additionally, the PVT receives dense afferents from several distinct hypothalamic areas (Matzeu et al., 2014), including orexininergic neurons important for the regulation of arousal and food reward (Hsu et al., 2014). Although it is known that the PVT is activated following salient experiences like drug exposure and stress (Bubser and Deutch, 1999; Penzo et al., 2015; Young and Deutch, 1998), the contributions of its afferents remain unresolved. Furthermore, whether durable memories are encoded by these salient experiences merits investigation.

In addition to fast neurotransmission, the PVT receives substantial piptidergic input, especially from regions of the hypothalamus. For example, the action of cocaine- and amphetamine-related transcript (CART) has been demonstrated to attenuate cocaine-primed reinstatement (James et al., 2010), potentially though its ability to suppress spontaneous transmission in the PVT (Yeoh et al., 2014). In contrast, PVT orexinergic transmission is thought to promote relapse (Hsu et al., 2014) in part through depolarization of PVT neurons (Ishibashi et al., 2005). These findings illustrate that PVT neuron activity plays a role in promoting drug-seeking, but raise many questions about the underlying molecular

and cellular specificity. Are all PVT neurons responsive to CART and orexins or are they distinguished by projection target? Which nuclei provide the neuropeptides, and at which synapses does CART inhibit fast neurotransmission? Do salient experiences in vivo alter the action of neuropeptides in the PV? These types of questions have not been asked in the PV, and future experiments will need to be directed at examining the function of fast neurotransmitters and neuromodulators in a mechanistic, circuit-directed approach.

2.2.2 Neuron Differentiation

There are several appropriate ways to tease apart PVT neuron function, however the approaches are not as straightforward as in the striatum and other limbic regions. For one the PVT is not believed to contain classical inhibitory interneurons, as expression of neither GABA nor parvalbumin was observed in the medial thalamus (Bentivoglio et al., 1991). Most PVT neurons are believed to express glutamate or aspartate as excitatory neurotransmitters (Arcelli et al., 1996; Christie et al., 1987), but many neurons in the region were found to contain neither. Some reports have found the expression of neuropeptides including enkephalin and galanin in the PVT (Arluison et al., 1994; Melander et al., 1986), but it remains to be tested whether these are co-expressed by glutamatergic projections or in some other population(s) of neurons. A combination of genetic and viral approaches, as well as targeted electrophysiology would be best suited to interrogating the function of potentially distinct PVT populations.

Another means of segregating PVT populations is through anatomy. For example, some PVT neurons project to the NAc, some project to the PFC, and some project to both (Otake and Nakamura, 1998). A dual retrograde viral approach could be taken to label each of these populations in order to examine functional differences in excitability, synaptic physiology, protein expression, and transcription. Examining the transcriptomics of PVT neurons based on anatomy is an exciting approach that could yield insight into PVT subpopulations. For example, ribosomal proteins tagged with HA can be introduced in a Cre-dependent manner and subsequently exploited for cell-type specific ChIP/qRT-PCR (Quintana et al., 2012). Driving Cre expression to target subpopulations defined by genetics (e.g. *Penk*-Cre) or projection target (e.g. NAc-RV-Cre) are promising avenues towards untangling PVT circuit function.

3. Conclusion

A growing hypothesis in the field, which this body of work supports, is that the striatal balancing act is not bidirectional for drug-related behaviors. That is, drugs of abuse exert more pronounced changes onto D1 MSNs, and changes in the A2A pathway may have little effect on drug-conditioned behaviors due to a lack of subsequent circuit-level changes. There are several non-exclusive potential mechanistic explanations. For one, while we and others have demonstrated the importance of striatal D1-NMDAR signaling, NMDARs may not generate the most prominent form of synaptic plasticity on A2A MSNs. In the NAc core and dorsal striatum, eCB-mediated plasticity has been documented on A2A MSNs, whereas

it is absent on D1 MSNs (Grueter et al., 2010; Kreitzer and Malenka, 2007). Cocaine-induced synaptic modifications may therefore proceed preferentially through NMDARs on D1 MSNs but through eCB signaling on A2A MSNs. Additionally, A2A MSNs may not play a major role in psychostimulant-related behaviors due to anatomy. A2A NAc MSNs send inhibitory projections primarily to the ventral pallidum (VP), but the VP also receives substantial innervation from D1 NAc MSNs (Kupchik et al., 2015). These projections may lie such that following psychostimulant administration, D1 MSN activity dominates the circuit-level logic and precludes small changes in A2A MSN function from being observed at the behavioral level. An exciting hypothesis is that VP neurons receiving projections from both MSN classes are involved in stimulant/locomotor-based behaviors, while those innervated strictly by A2A MSNs are involved in affective behaviors. Future experiments will need to be directed towards the targets of these A2A MSNs to examine the mechanistic basis of responses to stressful and reward-related stimuli. A combination of biochemistry and slice and in vivo electrophysiology, would be ideal to uncover how NMDAR signaling in each MSN population guides behavioral outcomes at the circuit level.

While the studies presented in this dissertation illuminate mThal-NAc projections as important for the persistent behavioral effects of cocaine exposure, much work needs to be continued to evaluate this circuitry in the regulation of long-term memories related to drug abuse and other motivational disorders. At mThal-D1(+) NAc core synapses, the metaplastic adaptations induced by cocaine facilitate GluN2C function and subsequent signaling. As its expression is limited in

the striatum and forebrain (Paoletti et al., 2013), modulation of GluN2C represents a potential novel treatment strategy for cocaine use disorders that may exert few off target effects. Additionally, because cocaine experience unmasks NMDAR-LTD at mThal-D1(+) NAc core synapses, a deep brain stimulation-like approach might be suitable for restoring synaptic function and generating beneficial behavioral outcomes without grossly affecting striatal circuity. Despite being largely overlooked in recent years, the mThal-NAc circuit clearly merits further investigation for its involvement in the pathophysiology of addiction and other psychiatric disorders. The circuits described in this dissertation provide an opportunity for the development of deep brain stimulation-like approaches to treat drug abuse disorders, and the unique molecular players identified could serve as viable targets for a pharmacological approach. In sum these studies simultaneously build upon the existing literature and drive the field forward towards a better understanding of addiction pathophysiology.

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