COCAINE-BASED SIGNALING CHANGES IN THE NUCLEUS ACCUMBENS, LATERAL HABENULA, AND THALAMUS

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ABSTRACT

The brain is an extraordinarily complex and organized system. Environmental information reaches the brain via the sensory systems, and this information is processed to interpret and make sense of the world. The mechanisms used to transmit information between neurons are also involved in directing and modifying the strength of these connections. Thus, the brain is always in a plastic state and has the ability to both interpret neural information and be shaped by it. Cocaine addiction is a progressive condition highlighted by maladaptive and compulsive behavior that develops after exposure to cocaine. Thus, cocaine exposure changes neural processing in the brain in ways that lead to the addicted state. The work presented here examines how neural circuits in addiction-related brain regions, such as those involved in motivated behavior and translating emotion into action, change at the cellular and molecular levels in response to cocaine exposure. The results uncover a variety of novel cocaine-induced changes in neural circuitry and processing which likely contribute to the development and/or maintenance of addiction.

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PREFACE

It is amazing for me to look back and see just how much I learned during the past 5 years. I was lucky that Yan believed that I could be molded and shaped into something resembling a competent graduate student (there was little to suggest such a thing at the time). Working with Yan has been a great learning experience. In addition to academic and declarative knowledge, many lessons learned relate to the type of grit and determination necessary to succeed not only in the current research environment, but also in life more broadly. I am happy to take these lessons with me as I start to enter the next phase of my professional life and beyond.

I have far too much to say about my experiences over the past 5 years to reasonably fit here, but I do want to convey my thanks to certain people for their support because it is important that I document their invaluable help and contributions to my development and, by extension, this work and future works that I am involved with. I have already mentioned Yan's paramount mentorship and guidance above. I would also like to thank Dr. Barb Sorg for being so patient with me and for being always willing to help; Dr. Yanhua Huang also for her patience and helpful guidance whenever it was needed; Dr. Oliver Schlüter for his guidance, support, and mentorship; Dr. Anna Suska for teaching and guiding me through the scary world of molecular biology; Dr. Susan Sesack and Joy Balcita-Pedicino for their helpfulness with tracing and anatomical experiments; my committee members Drs. Susan Sesack, Pat Card, Tony Grace, and Colleen McClung for keeping an eye on my training progress and keeping my goals both

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1.0 INTRODUCTION: THE BRAIN AND DRUG ADDICTION

The brain is an extraordinarily complex and organized system. It is also a system that is constantly changing. Countless signals are transmitted and biological processes occur at every moment. Environmental information reaches the brain via the sensory systems, and this information is heavily processed to make sense of and to decode the world. But, the information flowing in also has the ability to change the processing system. The mechanisms used to transmit information between neurons are also involved in directing and modifying the strength of these connections. Thus, the brain is always in a plastic state and has the ability to both interpret inputs and to be shaped by them. Momentary experiences may leave powerful impressions that can be remembered for entire life times. In this way, the brain's plasticity processes are both flexible and powerful. But, the complexity of the system has prevented us from fully understanding it.

Drug addiction is a progressive condition highlighted by maladaptive and compulsive behavior. Drug addiction exists as a matter of degree—the degree to which a person's thoughts and behaviors are affected by or preoccupied with taking and seeking addictive substances. Addiction is, therefore, a *cognitive* disorder of the brain which develops progressively from environmental exposure (to addictive drugs). There is commonly a physical dependence aspect to addiction, but addiction is more than physical dependence (Solomon and Corbit, 1974, Hyman and Malenka, 2001, Koob and Le Moal, 2001, Robinson and Berridge, 2003, Wise, 2004, Kauer and Malenka, 2007, Conrad et al., 2008). It would be relatively easy to treat addiction if it were

as simple as separating an addict from drugs through withdrawal until physical symptoms are gone. But, physical dependence to a substance is only the first challenge which must be overcome when trying to treat addiction. The cognitive challenge of dealing with cravings and the preoccupation with the substance remains long after physical dependence is overcome and the physical symptoms of withdrawal have subsided. In fact, it is the constant and persistent cravings during abstinence, which often escalate over time, that represent the biggest hurdles to overcome (Gawin and Kleber, 1986, Lu et al., 2004, Conrad et al., 2008, Wolf and Tseng, 2012). Thus, it is these cognitive barriers related to motivated behaviors which are the largest challenge in overcoming addiction.

The question is, then, how do addictive drugs create such strong cravings during withdrawal, even in the absence of further drug use? The well-accepted neuroadaptation theory states that addiction is the result of addictive substances acting on natural learning and memory processes in the brain (Hyman et al., 2006, Kauer and Malenka, 2007). Addictive drugs exploit these natural learning mechanisms, leading to strong and persistent memories related to drugs which then translate into cognition and behavior. However, the related neural systems which process thoughts and behaviors are incredibly complex and are not currently well understood. This thesis will explain why and how I have attempted to decode and examine parts of this complex neural system in an effort to better-understand how changes to these neural systems may lead to addiction in hopes of finding better treatments.

1.1 THE ANATOMY OF ADDICTION

Addiction is considered a cognitive disorder affecting emotions and motivations. Early work concerning motivated behaviors discovered that rats will learn a task to electrically self-stimulate parts of the brain (Olds, 1958). Rats will even work to self-stimulate despite other aversive consequences (Olds, 1958, Valenstein and Beer, 1962) and will starve themselves in favor of self-stimulation (Routtenberg and Lindy, 1965). These studies and others have confirmed that specific neural stimulation and signaling within the brain can mediate maladaptive and compulsive behaviors. Further studies examining this behavior have implicated the mesolimbic dopamine circuit between the VTA and NAc as a primary mediator (Fibiger et al., 1987, Wise and Bozarth, 1987).

1.1.1 The Mesolimbic System and Nucleus Accumbens

The mesolimbic circuit primarily involves the ventral tegmental area (VTA) and the nucleus accumbens (NAc; also known as the ventral striatum) along with the regions with which they send and receive signals. In this circuit, the NAc acts as a processing center for incoming emotional information from regions such as the amygdala, the hippocampus, and other regions before sending output to the ventral pallidum and the basal ganglia motor system (Mogenson et al., 1980, Kelley, 1999). This neural architecture positions the NAc as a critical gating point for the transition from emotions to actions, making it a central player in mediating motivations and goal-directed behavior including compulsive motivations and behaviors such as those observed in addiction.

Transmission from the VTA to the NAc is often thought of in terms of dopamine signaling. Dopamine sent from the VTA to the NAc has long been implicated in drug- and reward-seeking behavior. All drugs of abuse act either directly or indirectly by increasing dopamine signaling within the limbic circuit (Grace, 2000), and blocking dopamine signaling attenuates seeking behavior for both drug and natural rewards (De Wit and Wise, 1977, Wise et al., 1978, Gerber et al., 1981). Changes in dopamine signaling appear to be important for subsequent changes in glutamatergic signaling at NAc neurons (White, 1996, Wolf, 1998). And, these pathophysiological changes in excitatory glutamate signaling in the NAc are thought to underlie and maintain the addicted state (Wolf, 1998, Koob and Le Moal, 2001, Everitt and Robbins, 2005, Kalivas and Volkow, 2005).

The NAc is the ventral region of the striatum. It is composed of greater than 90% medium spiny neurons (MSNs) and 5-10% fast-spiking interneurons (Chang and Kitai, 1985, Kawaguchi et al., 1995, Meredith, 1999). As the principle neurons of the NAc, MSNs send GABAergic output from the NAc to motor-related systems important for the execution of motivated behaviors (Groenewegen et al., 1999, Kelley, 1999). MSNs have a low membrane resistance and generally require multiple excitatory inputs to fire action potentials (Wilson and Kawaguchi, 1996).

The NAc is divided into 2 subregions, the core and the shell. There is evidence that the core and shell have different roles. The core is generally involved with information related to motivationally salient environmental stimuli, whereas the shell is more involved in unconditioned responses—though these two subregions are not entirely independent and information is transmitted between them regularly (Meredith et al., 2008, Wolf and Ferrario, 2010). Though both subregions share most inputs, there is often a bias for one subregion over the

other. Subtle difference in connectivity and microarchitecture lead to different roles in processing information for the core and shell (Meredith et al., 1992, Maldonado-Irizarry and Kelley, 1994). In general, the core has a larger role in mediating learned and conditioned behaviors while the shell has a stronger role in mediating responses to unconditioned stimuli (Meredith et al., 2008). Interestingly, the morphological differences between MSNs in the core and shell also appear to make the shell more labile in terms of dendritic growth and formation, whereas the core is more labile in terms of traditional NMDA-mediated long-term potentiation/depression (Meredith et al., 2008).

1.1.2 Nucleus Accumbens Inputs

The NAc receives extensive glutamatergic inputs from many brain regions including the hippocampus, the basolateral amygdala (BLA), and the medial prefrontal cortex (mPFC) (Mogenson et al., 1980, Groenewegen et al., 1999). It is the combined excitatory and inhibitory input processed by NAc MSNs which determines NAc functional output. We will focus on the excitatory glutamatergic inputs to NAc MSNs, as glutamate is the primary driver of action potentials and functional output.

The hippocampus sends and receives information related to environmental context, and is involved in drug seeking behavior, specifically context-induced reinstatement of drug seeking (Vorel et al., 2001, Sun and Rebec, 2003, Fuchs et al., 2005).

BLA neurons project to the NAc and encode emotional significance and motivational information. BLA neurons are activated only when a contextual cue is paired with a conditioned stimulus (Rosenkranz and Grace, 2002, Tye and Janak, 2007). And, when the reward association with a CS is reversed, the neural response of the BLA is also reversed (Schoenbaum et al., 1999).

This indicates that BLA output is related to emotional salience. BLA-to-NAc signaling has also been shown to mediate reinstatement of drug seeking and craving during withdrawal (Lee et al., 2013).

The mPFC also sends major glutamatergic input to the NAc. The mPFC is largely thought to relay information related to goal-directed/motivated and planned/executive behavior (Kalivas and Volkow, 2005). The infralimbic mPFC biases its projections towards the NAc shell, while the prelimbic mPFC mostly sends its projections to the NAc core (Krettek and Price, 1977, Sesack et al., 1989). These different projections have opposite effects on drug seeking behavior. Following exposure to cocaine, the infralimbic projections to the shell appear to facilitate drug seeking during withdrawal while the prelimbic projections to the core appear to inhibit drug seeking (Ma et al., 2014).

Together, these and other pathways send their signals to the NAc. The NAc then receives a variety of incoming information related to context, emotional salience, the value of rewards, and other impulses, which is then processed within NAc MSNs and drives neural output (O'Donnell and Grace, 1995). Thus, changes in the strength and disruptions in the balance of incoming signals to the NAc (or to how the NAc processes these signals) can lead to alterations in the output sent downstream to motor-related regions including the ventral pallidum and basal ganglia.

1.2 COCAINE AND SYNAPTIC PLASTICITY

The neuroadaptaion theory posits that drug addiction is the result of addictive substances acting on natural learning and memory processes in the brain to create addiction-related memories (Hyman, 1996, Hyman and Malenka, 2001, Hyman et al., 2006). This is achieved when drug exposure induces plastic changes within the neurons and circuits which mediate motivated behavior, cognition, and emotion. Thus, alterations in circuitry and connections related to the NAc are thought to underlie drug addiction (Everitt and Robbins, 2005, Kalivas and Volkow, 2005). Via this mechanism of drug-induced plasticity, addiction can be considered a form of memory—a memory which is long-lasting, durable, and difficult to weaken (Lee and Dong, 2011). Understanding how neural signaling changes in response to cocaine will help to reveal how the information is encoded and processed and may provide targets for the treating of addiction.

1.2.1 Cocaine Pharmacology

Cocaine is a psychostimulant which acts by blocking the function of the dopamine transporter (DAT) protein. By binding with and blocking this transporter, dopamine cannot be cleared from the synaptic space and extracellular areas around neurons. This extends the ability of dopamine to be an active signaling molecule and leads to increased levels of dopamine signaling. This acute pharmacological effect of cocaine also leads to a cascade of cellular changes related to mesolimbic circuit function and glutamatergic signaling (Jones et al., 2000, Thomas et al., 2000, Ungless et al., 2001, Saal et al., 2003).

1.2.2 Glutamate Receptors: AMPARs and NMDARs

As the primary excitatory neurotransmitter throughout the brain, glutamate has the strongest direct influence over action potential firing and the output of neural signals. There are 2 major receptor types which are activated by glutamate, 1) a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors (AMPARs) and 2) N-methyl-D-aspartate (NMDA) receptors (NMDARs).

AMPARs are the most common type of receptor found throughout the nervous system and mediate the majority of excitatory synaptic transmission throughout the brain. AMPARs are ionotropic transmembrane receptors that open their channel when glutamate binds, allowing Na^+ to flow into the cell (and K^+ to flow out) and depolarizing the postsynapse (Chater and Goda, 2014). AMPARs are tetramers composed of two pairs of four possible subunits (GluA1-4, also referred to as GluR1-4) (Hollmann and Heinemann, 1994). GluA2 subunits are the most commonly found of these subunits and their presence causes the ion channel to be impermeable to Ca^{2+} ions (Greger et al., 2003). AMPARs that lack GluA2 subunits allow both Na^+ and Ca^{2+} ions to pass through their channel into the cell when bound by glutamate and are called calcium-permeable AMPARs (CP-AMPARs). CP-AMPARs have a higher single channel conductance and faster rise and decay kinetics compared to GluA2-containing AMPARs (Swanson et al., 1997). The ability of CP-AMPARs to allow extracellular calcium into the postsynapse leads to interesting implications related to synaptic plasticity (see below for more). AMPARs are also highly dynamic. They have a high trafficking rate to and from the cell membrane and have high lateral mobility along the cell membrane, allowing them to traverse between synaptic to extrasynaptic locations (Nishimune et al., 1998). Persistent changes in the number of stable AMPARs at postsynaptic locations influences the efficacy of glutamate signal transduction

(Luscher et al., 1999, Chater and Goda, 2014), a process referred to as long-term potentiation (LTP) or long-term depression (LTD), which increases or decreases the efficiency of glutamate signal transduction, respectively.

Similar to AMPARs, NMDARs are also tetrameric ionotropic transmembrane glutamate receptors that contain two pairs of four possible subunits (NR1-4, also known as GluN1-4). However, NMDARs have other characteristics which make them different as well. For one, NMDARs require glycine (or D-serine) as a co-agonist with glutamate in order to open their channel (Kleckner and Dingledine, 1988). Additionally, NMDARs are voltage-gated: the ion channel is blocked by Mg^{2+} (or Zn^{2+}) ions at hyperpolarized resting potentials but is released at depolarized potentials (Jahr and Stevens, 1990). Thus channel blockade by Mg^{2+} ions essentially makes NMDARs non-functional at resting cell potentials. Glutamate and glycine may bind to NMDARs under normal resting potentials, but the ion channel remains nonconductive due to Mg^{2+} blockade. NMDARs thus require the presence of other agents such as AMPARs to create a depolarized environment so that the Mg^{2+} block is released and the channel becomes conductive. Under these functional conditions, NMDARs are non-selectively conductive to cations, meaning that positive ions, including extracellular Ca^{2+}, may pass through NMDARs into the cell.

Calcium conductance is an important characteristic of NMDAR function due to the role of Ca^{2+} signaling within neurons. The presence of extracellular calcium can trigger additional calcium release from intracellular stores and mediates signal-transduction cascades which lead to lasting changes such as alterations in AMPAR trafficking and synaptic potentiation and depression (Morris, 2013). Calcium influx through NMDARs activates a protein in complex known as Calcium/Calmodulin-dependent protein kinase II, an important interaction for initiating LTP processes (the increase in stable synaptic AMPARs) and memory formation (Coultrap and

Bayer, 2012). Blocking NMDARs blocks the induction of LTP at synapses (Collingridge et al., 1983) and prevents behavioral learning and memory encoding (Morris et al., 1986, Morris, 2013). In these ways, the joint function of AMPARs and NMDARs is important not only for sending neural signals between cells, but also for directing the plasticity of circuits through which neural signals are sent.

1.2.3 The Role of Glutamate in Cocaine Addiction

All drugs of abuse, including cocaine, either directly or indirectly increase dopamine signaling within the limbic circuit (Grace, 2000), but glutamatergic signaling is thought to be the primary mediator responsible for the maintenance of addiction to cocaine and other drugs (Wolf, 1998, Koob and Le Moal, 2001, Fuchs et al., 2005, Kalivas and Volkow, 2005, Kauer and Malenka, 2007). Glutamate transmission onto postsynaptic AMPARs and NMDARs is the primary way to synaptically communicate between neurons. Thus, in many ways, changes in the number or function of these receptors fundamentally changes the neural information sent between cells. And, changes in glutamate transmission in the mesolimbic circuit have been critically implicated in mediating cocaine relapse and cocaine seeking behavior (McFarland and Kalivas, 2001, Everitt and Robbins, 2005, Kalivas and Volkow, 2005). Results from many animal models of addiction show that AMPAR activation within the NAc is critical for cocaine seeking. Intra-NAc infusion of AMPAR antagonists blocks cue-induced cocaine seeking during withdrawal (Conrad et al., 2008), cue-induced reinstatement (Backstrom and Hyytia, 2007), and cocaine-induced reinstatement (Cornish and Kalivas, 2000). Likewise, infusion of AMPA into the NAc reinstates cocaine seeking behavior after extinction training (Cornish et al., 1999, Suto et al., 2004). Cocaine also induces an increase in the amount of AMPARs at NAc synapses that begins about

3-4 days after exposure and lasts for weeks afterwards (Boudreau and Wolf, 2005, Kourrich et al., 2007). Re-exposure to cocaine after a period of withdrawal (cocaine challenge) transiently decreases AMPAR surface expression, and further withdrawal shows that AMPARs return back to their elevated levels again afterwards (Bachtell and Self, 2008). An increase in synaptic and/or membrane associated AMPARs indicates that LTP-like processes have occurred and that excitatory signal transduction is increased. It seems likely that this increase in synaptic excitability may correspond to greater sensitivity of NAc synapses to input related to cocaine cues and stimuli. Thus, the upregulation of AMPARs may be responsible for the sensitization of incentive salience to cocaine (Wolf and Ferrario, 2010, Wolf and Tseng, 2012).

AMPARs inserted at NAc neurons 1-3 weeks after cocaine exposure are almost exclusively AMPARs containing GluA1 and 2 subunits (Boudreau et al., 2007, Kourrich et al., 2007). However, after 35 days of withdrawal from cocaine, CP-AMPARs (lacking GluA2 subunits) appear in the NAc (Conrad et al., 2008). CP-AMPARs are not normally present. Glutamate still activates CP-AMPARs like normal GluA2-AMPARs, but the channel conductance is higher (Liu and Zukin, 2007). Thus NAc MSNs may be even more sensitive to glutamate signals after longer withdrawal from cocaine (Wolf, 2010).

Withdrawal from cocaine induces a phenomenon known as the incubation of cocaine craving, in which cocaine-seeking behavior by animals previously exposed to cocaine progressively increases over time throughout withdrawal (Grimm et al., 2001, Lu et al., 2004). Multiple studies have shown that the appearance and presence of CP-AMPARs during withdrawal corresponds to drug-seeking behavior during cue-induced re-exposure (Conrad et al., 2008), and specifically that stimulation-induced removal of CP-AMPARs reduces the incubation of cocaine craving (Lee et al., 2013, Ma et al., 2014).

1.2.4 Silent Synapses

Silent synapses are glutamatergic synapses which contain NMDARs but have no stable AMPARs (Isaac et al., 1995, Liao et al., 1995). Normal activation of these synapses with glutamate results in no postsynaptic conductance because without nearby AMPARs to depolarize the cell in response to glutamate, NMDAR channels remain inactive due to Mg^{2+} block (see section 1.2.2 for more information). Silent synapses are thought to be immature synaptic connections which do not have stable AMPARs at the postsynaptic membrane (Groc et al., 2006, Kerchner and Nicoll, 2008, Hanse et al., 2013). Silent synapses are found at high levels in young and juvenile brains (Kerchner and Nicoll, 2008, Sametsky et al., 2010) and the number of silent synapses drops significantly as the brain matures (Durand et al., 1996, Isaac et al., 1997). These silent synapses may be either strengthened or weakened based on different patterns of activity (Isaac et al., 1997, Hanse et al., 2013). Therefore, silent synapses act as efficient substrates for plasticity via AMPAR insertion at the postsynaptic membrane.

Cocaine also induces generation of silent synapses in the NAc of mature animals (Huang et al., 2009, Brown et al., 2011). However, there has been some debate over how these silent synapses form. In theory, cocaine-generated silent synapses in the NAc could form 1) via generation of new synaptic contacts with the insertion of NMDARs or 2) via removal and internalization of AMPARs at existing synapses. Evidence from our lab and others points to the idea that cocaine-generated silent synapses are the result of de novo synapse creation. This evidence comes from several sources: 1) cocaine-generated silent synapses largely contain NR2B NMDARs, as newer synaptic contacts usually do before being swapped for NR2A NMDARs (Huang et al., 2009); 2) NMDARs composed of newly constructed subunits are incorporated at silent synapses (Huang et al., 2009), 3) AMPAR surface expression largely stays

constant during repeated cocaine exposure at times when silent synapses are forming (Boudreau and Wolf, 2005), 4) cocaine increases the frequency of miniature EPSCs in NAc MSNs with no indication of presynaptic changes (Kourrich et al., 2007), and 5) drugs of abuse including cocaine persistently increase the number of spines and dendritic connections within the NAc (Robinson and Kolb, 2004). Others have suggested that silent synapses may be a byproduct of synaptic scaling processes in the NAc, whereby AMPARs are removed from MSN synapses after being persistently activated by cocaine to normalize activity within the circuit (Koya et al., 2012). One of the difficulties contributing to this debate is the fact that methods used to detect silent synapses are relative to the number of functional synapses. Thus, generation of silent synapses either via new synaptic contacts or via existing synapses would show similar results in tests for silent synapses because the total number of synapses is unknown.

Regardless of how they are formed, the apparent ability of silent synapses to undergo efficient LTP processes based on the insertion of AMPARs (Isaac et al., 1997, Kerchner and Nicoll, 2008) creates a situation where there are highly plastic substrates in the NAc after cocaine exposure. Recently, our lab has connected the appearance and maturation of silent synapses at synaptic connections in the NAc with the appearance of CP-AMPARs (Lee et al., 2013, Ma et al., 2014). However, not all silent synapses in the NAc have been observed to attract CP-AMPARs (Ma et al., 2014). Thus, it appears that withdrawal from cocaine differentially affects glutamatergic afferents to the NAc and that maturation of silent synapses may be different among afferents to the NAc as well. Understanding this differentiation in plasticity between glutamatergic inputs to the NAc may be crucial to understanding how cocaine exposure and withdrawal lead to an addicted state.

1.3 DYNORPHINS AND THE NUCLEUS ACCUMBENS

In addition to glutamatergic and dopaminergic signaling, several other intercellular signaling mechanisms are present in the NAc (Meredith, 1999, Hyman et al., 2006). Dynorphins are a type of signaling molecule found in the NAc which may be particularly relevant in addiction. Dynorphins are a family of small signaling peptides that are all derived from a common precursor, prodynorphin (Kakidani et al., 1982). From this precursor, comes dynorphin A (DynA, 17 and 8 amino acids), dynorphin B (DynB, 13 amino acids), and several other varieties (Healy and Meador-Woodruff, 1994). DynA and DynB are produced and released locally by NAc MSNs. Dynorphins bind to and activate opioid receptors, including kappa, mu, and delta. Both DynA and B are endogenous agonists of kappa opioid receptors, though their selectivity for the kappa variant over other opioid receptors is relatively weak (Corbett et al., 1982). Activation of either kappa or mu opioid receptors in the NAc acutely decreases excitatory post-synaptic currents (EPSCs) in MSNs, an effect that appears to be mediated by presynaptic action of the opioid receptor in both cases (Dhawan et al., 1996, Hjelmstad and Fields, 2003).

It has been suggested that NAc dynorphin signaling significantly contributes to the stress and depressed emotional states present during drug withdrawal (Shirayama et al., 2004, Hauser et al., 2005). Exposure to stress and addictive drugs such as cocaine increase the expression of prodynorphin in the NAc, and induced dynorphin signaling within the NAc leads to aversive behaviors (Shippenberg et al., 2007). And, elevated dynorphin levels have been implicated in several NAc-based behavioral changes (Shippenberg et al., 2007, Shippenberg, 2009). Thus, it seems possible that dynorphins may play a role in the stress and negative affect experienced after withdrawal from drugs. It is these negative feelings which can often cause relapse, as addicts seek drugs to mitigate this negative affect (Koob et al., 2014). It is with these ideas in mind that

the investigation into how dynorphin signaling changes in the NAc after cocaine exposure was undertaken (detailed in Chapter 2).

1.4 THE LATERAL HABENULA AND ADDICTION

Similar to effects of dynorphin signaling, the lateral habenula (LHb) brain region has been strongly implicated in contributing to motivated behaviors by mediating negative rewards and aversive behavior (Lecourtier and Kelly, 2007, Hikosaka, 2010). The habenula is part of the epithalamic region, and is divided into the medial and lateral nuclei. The LHb consists of many further subdivisions with highly heterogenous populations of cells (Andres et al., 1999, Weiss and Veh, 2011, Aizawa et al., 2012). However, LHb neurons also share many characteristics. LHb neurons primarily send glutamatergic outputs and have a notable ability to generate rebound action potentials following periods of hyperpolarization (Chang and Kim, 2004, Li et al., 2011, Weiss and Veh, 2011).

The connectivity of the LHb makes it a prime candidate for influencing rewards and motivated behavior. The LHb receives glutamatergic input from regions such as the lateral hypothalamus, the VTA, and the prefrontal cortex while receiving GABAergic inputs from various regions including the VTA, NAc, and pallidum (Araki et al., 1988, Lecourtier and Kelly, 2007, Bianco and Wilson, 2009, Sesack and Grace, 2010, Aizawa et al., 2012, Shabel et al., 2012). Interestingly, while it has been known that VTA fibers that contain dopamine markers also project to the LHb, it has recently been found that these fibers actually release GABA and not dopamine (Stamatakis et al., 2013). However, dopamine does appear to have a role in signaling to the LHb (Good et al., 2013, Jhou et al., 2013). These studies and others also show

clear evidence for the organization of microcircuits within the LHb, which appears to correspond to the heterogeneity of LHb neurons (Maroteaux and Mameli, 2012).

Glutamatergic outputs from the LHb are sent to regions including the VTA and the rostromedial tegmental nucleus (RMTg) (Ji and Shepard, 2007, Lecourtier et al., 2008, Balcita-Pedicino et al., 2011). The RMTg is a GABAergic nucleus which sends projections to dopamine cells in the VTA (Jhou et al., 2009, Balcita-Pedicino et al., 2011). Thus, the excitatory output sent from the LHb can transform into an inhibitory influence to VTA dopamine neurons via the RMTg, and LHb neuronal activity has been shown to inhibit the dopamine neurons in the VTA (Ji and Shepard, 2007, Matsumoto and Hikosaka, 2007, Hikosaka, 2010). Additionally, optogenetic activation of LHb terminals appears to evoke EPSCs at GABAergic neurons in either the VTA or RMTg, and not in VTA dopamine neurons (Stamatakis et al., 2013).

This ability of the LHb to mediate aversive behavior and negative affect via the inhibition of VTA dopamine neurons makes the LHb an intriguing target for addiction research because it is a convergence point for reward circuits and aversive opponent processes (Lammel et al., 2012). Cocaine exposure initiates negative feelings and dysphoria that persist beyond the acute euphoric effects, and it is the desire to mitigate these prolonged negative feelings which often drives addicts to continue to seek drugs (Solomon and Corbit, 1974, Koob and Le Moal, 2001, Koob et al., 2014). In fact, the LHb exhibits greater activation during depressed like states (Li et al., 2011, Li et al., 2013), and deep brain stimulation in the LHb has been shown to affect cocaine-seeking behavior (Friedman et al., 2010, Lax et al., 2013). Cocaine-block of the dopamine transporter, densely expressed in the LHb, may lead to dopamine-induced changes in LHb signaling (Vaughan and Foster, 2013). Following cocaine exposure, LHb neurons projecting to the RMTg showed a preference for increased activity based on Fos activation (Jhou et al., 2013).

Additionally, synaptic transmission from LHb projections to RMTg neurons is enhanced after cocaine, but not in LHb projections to the VTA (Maroteaux and Mameli, 2012). Collectively, this data shows that the LHb is involved in mediating aversive behaviors and negative affect while also influencing drug seeking behavior. However, much information is missing from the full story explaining the LHb's influence over aversive feelings and drug-seeking behavior (Lecca et al., 2014). The experiments detailed in Chapter 3 sought to uncover details corresponding to how cocaine self-administration affects alterations in LHb signaling

1.5 THE PARAVENTRICULAR NUCLEUS OF THE THALAMUS AND ADDICTION

One of the lesser-examined but major sources of glutamate input to the NAc is the paraventricular nucleus of the thalamus (PVT). The PVT is a subnucleus of the thalamus and lies on the midline just ventral and adjacent to the third ventricle. The PVT sends glutamatergic projections to the NAc, central amygdala, VTA, and mPFC (Van der Werf et al., 2002, Smith et al., 2004), positioning it well to mediate corticostriatal signaling involved with motivation and reward (Berendse and Groenewegen, 1990, O'Donnell et al., 1997, Otake and Nakamura, 1998, Cardinal et al., 2002, Parsons et al., 2007, Vertes and Hoover, 2008). A large percentage of PVT neurons are branched, meaning that a single PVT neuron can signal to multiple regions at once (Otake and Nakamura, 1998). PVT connections to the NAc often synapse next to incoming dopamine fibers, making it likely that dopamine signaling is able to directly modulate PVT inputs in the NAc (Pinto et al., 2003).

The PVT has been shown to be involved in cocaine seeking behaviors. Specifically, inactivating or lesioning the PVT blocks cocaine-primed reinstatement (James et al., 2010), sensitization to cocaine (Young and Deutch, 1998), and expression of cocaine-induced conditioned place preference (Browning et al., 2014). Moreover, presentation of cocaine-associated cues activates PVT neurons based on Fos examination (Brown et al., 1992). The PVT appears to be specifically activated by conditioned rewards and reward cues and is not activated by unconditioned stimuli (Brown et al., 1992, Wedzony et al., 2003, Matzeu et al., 2014). The experiments detailed in Chapter 4 examine the signaling characteristics of the PVT-to-NAc glutamatergic pathway and how this pathway changes in response to cocaine self-administration.

One additional interesting aspect of the PVT is that it expresses a high density of orexin (also known as hypocretin) receptors (Kirouac et al., 2005). Orexin is a signaling neuropeptide produced exclusively in the hypothalamus and projected to various regions throughout the brain, including the PVT, VTA, and NAc shell (Peyron et al., 1998). Studies have primarily focused on the role of orexin signaling as it relates to natural rewards such as food, but orexin signaling also modulates addictive behavior (Baimel and Borgland, 2012, Mahler et al., 2012, Matzeu et al., 2014). Drug-associated cues activate orexin neurons in the lateral hypothalamus, and stimulating orexin neurons reinstates extinguished drug-seeking behavior (Harris et al., 2005). Orexin fibers from the hypothalamus target the PVT and may stimulate PVT-to-NAc glutamatergic transmission.

1.6 SUMMARY

Action potential firing between neurons represents the basic from of information transfer of neural information—a single bit of data sent from one cell to another for processing. The processing of this data occurs within single neurons as a complex spatial, temporal, and biological function involving the state of the neuron and the summation of all incoming signals. This physiological activity is processed within single neurons and determines whether a neuron reaches its threshold of excitation necessary to fire its own action potential to connected cells. These physiological calculations are repeated indefinitely at billions of neurons across the brain at every moment, ultimately leading to the functional output of the brain—all the conscious and unconscious thought processes and behaviors of an organism. It's no wonder that decoding this neural code is a difficult task. However, the brain can be divided into smaller, easier to understand portions; we examine one process at a time or one brain region at a time, and then try to piece the full picture back together after collecting enough pieces, hoping that the pieces fit together. Though this methodology can lead to problems, it continues to provide the best insights into the incredibly complex neural code that underlies the thoughts and behaviors of all organisms.

Emotions, motivations, and reward-related behaviors are an important part of proper functioning in complex organisms. Appropriately, a large number of brain regions and neurons are involved with processing the complex sets of neural data (involving emotional salience, current state, conditioned stimuli, context, reward value, etc.). Electrophysiology and patch-clamping techniques provide the ability to examine signal processing at the level of single

neurons. Patch-clamping can reveal cellular and molecular properties of informational processing in neurons and can also be used to reveal changes in these processing properties.

Addiction is a disorder which develops after exposure to addictive drugs. This situation provides a clear trigger stimulus responsible for the development of addictive behaviors: exposure to the addictive drug. Based on the evidence reviewed to this point, it is clear that cocaine exposure induces cellular and molecular changes in the mesolimbic circuit and related regions, which affect normal processing and lead to addiction. Examining specifically where and how these neural processing changes occur in the brain after exposure to addictive drugs is important for: 1) identifying which neural circuits are affected and may contribute to addiction-related processing changes; 2) determining how the circuits change, providing a comparison between normal and drug-induced neural functioning; and 3) indicating potential targets for manipulation to reverse or treat the changes in neural processing.

In the first series of experiments (Chapter 2), we examine dynorphin signaling in the NAc after cocaine and saline exposure. A noncontingent model of cocaine exposure is used, and the pharmacological effects of dynorphin signaling on NAc MSN neuron activity are examined. We find that that cocaine exposure leads to an increase in dynorphin signaling for both dynorphin A and B, which reduces synaptic signals to NAc neurons via presynaptic mechanisms. We also find that this reduction in synaptic transmission to MSNs occurs via kappa opioid receptors for dynorphin A, but dynorphin B signaling occurs through kappa- and mu-independent mechanisms.

In the second series of experiments (Chapter 3), we examine the excitability of lateral habenula neurons after cocaine and saline self-administration. It was found that cocaine self-administration transiently increases the excitability of lateral habenula neurons for at least 7 days

after cocaine training, but that excitability returns to baseline by day 45 of withdrawal from cocaine. There is a corresponding increase in membrane resistance, which appears to mediate the increase in excitability of these neurons during this time. Other measurements looking for potential changes in calcium-activated potassium channels and sodium channels found no differences at any point.

In the third series of experiments (Chapter 4), we examined the signaling characteristics of the glutamatergic pathway from the paraventricular nucleus of the thalamus to the nucleus accumbens after cocaine and saline self-administration. This pathway was isolated by expressing channelrhodopsin in paraventricular thalamic neurons and using optical stimulation. Several characteristics of this pathway were described, including a high basal level of silent synapses and the presence of calcium-permeable AMPARs. Cocaine self-administration affected both pre and postsynaptic properties of this pathway by increasing silent synapse levels and increasing the probability of presynaptic transmitter release. Long-term withdrawal revealed several additional changes within this pathway including a reduction in the number of synaptic release points, a strengthening of remaining synapses based on an increase of quantal size, and a return in silent synapses back to baseline levels. Additionally, we found that blocking AMPAR internalization in MSNs prevented cocaine-generation of silent synapses within this pathway and lowered baseline levels as well.

The results from these experiments reveal specific cocaine-induced molecular and cellular changes to signaling properties at brain regions related to addiction and motivation. Thus, these changes in signaling properties likely contribute to maladaptive neural processing following cocaine exposure and ultimately contribute to addiction-related thoughts and behaviors.

2.0 EXPOSURE TO COCAINE ALTERS DYNORPHIN-MEDIATED REGULATION OF EXCITATORY SYNAPTIC TRANSMISSION IN NUCLEUS ACCUMBENS NEURONS[1]

2.1 OVERVIEW AND INTRODUCTION

Background: Dysregulation of excitatory synaptic input to nucleus accumbens (NAc) medium spiny neurons (MSNs) underlies a key pathophysiology of drug addiction and addiction-associated emotional and motivational alterations. Dynorphin peptides, which exhibit higher affinity to κ type opioid receptors, are upregulated within the NAc upon exposure to cocaine administration, and the increased dynorphin-signaling in the NAc has been critically implicated in negative mood observed in cocaine- or stress-exposed animals. Despite such apparent behavioral significance of the NAc dynorphins, the understanding of how dynorphins regulate excitatory synaptic transmission in the NAc remains incomplete.

Methods: We used electrophysiological recording in brain slices to examine the effects of dynorphins on excitatory synaptic transmission in the NAc.

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Results: We focused on two key dynorphins, dynorphin A and B. Our current results show that dynorphin A and B differentially regulated excitatory postsynaptic currents (EPSCs) in NAc MSNs. Whereas perfusions of both dynorphin A and B to NAc slices decreased EPSCs in MSNs, the effect of dynorphin A but not dynorphin B was completely reversed by the κ receptor-selective antagonist nor-binaltorphimine. These results implicate κ receptor-independent mechanisms in dynorphin B-mediated synaptic effects in the NAc. Furthermore, repeated exposure to cocaine (15 mg/kg/day via intraperitoneal injection for 5 days, with 1, 2, or 14 days withdrawal) completely abolished dynorphin A-mediated modulation of EPSCs in NAc MSNs, whereas the effect of dynorphin B remained largely unchanged.

Conclusions: Given the quantitatively higher abundance of dynorphin B in the NAc, our present results suggest that the dynorphin B-mediated, κ receptor-independent pathways predominate in the overall effect of dynorphins in cocaine-pretreated animals and potentially in cocaine-induced alterations in mood.

The nucleus accumbens (NAc) has long been hypothesized as a key brain site that mediates emotional and motivational responses (Kelley, 2004a, b). Medium spiny projection neurons (MSNs) contribute to >90% of the neuronal population within the NAc (Meredith, 1999). Malfunction of these NAc MSNs underlies a key pathophysiology of emotional and motivational distortions associated with cocaine addiction (Kelley, 2004a, b). The functional output of NAc MSNs heavily relies on excitatory synaptic input; it is the synchronous excitatory synaptic inputs that drive MSNs into functionally active states in which MSNs execute their actions by firing action potentials (O'Donnell and Grace, 1995). Excitatory synaptic transmission to NAc MSNs is regulated by a myriad of neuromodulator systems. Distortion of these regulations is presumably one way in which pathogenic stimulations, such as exposure to cocaine

or stress, reshape the functional output of NAc, resulting in emotional and motivational alterations (Kelley, 2004a, Hyman et al., 2006).

Among the neuromodulator-based regulation of NAc MSNs, dynorphin-signaling is particularly important for stress- or addiction-associated negative mood (Shippenberg et al., 2007, Bruchas et al., 2010). Dynorphins comprise a family of biologically active peptides derived from the common precursor prodynorphin (Kakidani et al., 1982) and are enriched in the NAc (Healy and Meador-Woodruff, 1994). Exposure to stress or drugs of abuse upregulates the gene expression of prodynorphin in the NAc, and experimental upregulation of NAc dynorphin-signaling produces aversive behaviors (Shippenberg et al., 2007). Despite our understanding of the behavioral effects of dynorphins, much less in known about how dynorphins regulate excitatory synaptic transmission in the NAc.

The two key dynorphins derived from prodynorphin are dynorphin A (DynA) and B (DynB). Early immunohistochemical results from the NAc and dorsal striatum show that DynB is more abundant than DynA (Healy and Meador-Woodruff, 1994). Additionally, both DynA and DynB are thought to be endogenous agonists of κ receptors on the basis of affinity studies, although their selectivity for κ receptors over other opioid receptors is poor (Corbett et al., 1982). Using a more selective κ receptor agonist (U69593), recent studies demonstrated that activation of κ receptors acutely decreases excitatory postsynaptic currents (EPSCs) in NAc MSNs, an effect likely mediated by inhibition of presynaptic glutamate release (Hjelmstad and Fields, 2001). However, it is not clear whether this effect holds for DynA and DynB and whether this κ receptor-mediated synaptic modulation is affected by dynorphin-associated pathophysiological conditions. Here we demonstrated that, similar to U69593, DynA inhibited EPSCs in NAc

MSNs, an effect that could be reversed by κ receptor-selective antagonist nor-binaltorphimine (nor-BNI). In contrast, although DynB also inhibited EPSCs in NAc MSNs, this effect was not completely reversed by nor-BNI, suggesting a κ receptor-independent mechanism. Moreover, after repeated exposure to cocaine, the effect of DynA on EPSCs in NAc MSNs was abolished, whereas the effect of DynB was still present. Therefore, DynA and DynB might use different cellular and molecular mechanisms in regulating excitatory synaptic transmission to NAc MSNs and thus might differentially regulate NAc-based emotional and motivational responses.

2.2 MATERIALS AND METHODS

2.2.1 Animal Use

Male Sprague–Dawley rats at an age of 22–24 days were allowed to acclimate to their home-cage (housed individually) for 5–7 days with free access to food and water under a 12:12-hour light/dark cycle. Rats were then either kept in the home cage for an additional 7 days or received cocaine/saline administration for 5 days, followed by a 1–2-day withdrawal period. Rats at 35–40 days (with or without cocaine treatment) were used for electrophysiological recording.

2.2.2 Intraperitoneal Injection of Cocaine

We used a 5-day procedure of repeated cocaine administration, which was similar to earlier studies (Huang et al., 2009, Ishikawa et al., 2009, Mu et al., 2009). Briefly, rats received one intraperitoneal injection (IP) of (-)cocaine hydrogen chloride (15 mg/kg) or the same volume of

saline/day for 5 days. Injections were performed within the home-cage at approximately 9:00 am each day. Treated rats were then killed for electrophysiological recordings 1–2 days or 2 weeks after the last injection.

2.2.3 NAc Slice Preparation, Cell Selection, and Electrophysiology

Detailed procedures for obtaining NAc slices can be found in our previous publications (Dong et al., 2006a, Huang et al., 2008, Lee et al., 2008, Ishikawa et al., 2009). Briefly, coronal NAc slices of 250–300-μm thickness were cut such that the preparation contained the signature anatomical landmarks that delineated the NAc subregions. Slices were submerged in a recording chamber and were continuously perfused with regular oxygenated artificial cerebrospinal fluid (in mmol/L: 126 sodium chloride, 1.6 potassium chloride, 1.2 sodium dihydrogen phosphate, 1.2 magnesium chloride, 2.5 calcium dichloride, 18 sodium bicarbonate, and 11 glucose, 295–305 mOsm, equilibrated at 31–34°C with 95% oxygen/5% carbon dioxide).

Electrophysiological recordings were preferentially made from the MSNs located in the ventral-medial subregion of the NAc Shell (referred to as NAc MSNs in this study). Standard whole-cell recordings were made with a MultiClamp 700B amplifier (Molecular Device, Foster City, California) through an electrode (2–6 mΩ) in all electrophysiological experiments. Voltage-clamp recordings were used to measure EPSCs and inhibitory postsynaptic currents (IPSCs) in NAc MSNs. The intracellular and extracellular solutions used can be found in our published papers for EPSC (Dong et al., 2006a, Huang et al., 2008) and IPSC (Dong et al., 2006b, Tyszkiewicz et al., 2008) recording. To record EPSCs and miniature excitatory postsynaptic currents (mEPSCs), the extracellular solution routinely contained picrotoxin (.1 mmol/L) to block γ-aminobutyric acid–A receptor-mediated currents. To record IPSCS, the

extracellular solution contained 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 5 μmol/L) and D(-)2-amino-5-phosphonovaleric acid (D-APV; 50 μmol/L) to block α-amino-3-hydroxy-5-methylisoxazole propionate receptor- and *N*-methyl-D-aspartate receptor-mediated currents. For evoked EPSCs and IPSCs, presynaptic stimuli (intensity, 200–500 μA; duration, 300–600 μs; frequency, .1 Hz) were applied through a monopolar microelectrode. The stimulating electrode was placed close to recorded neurons (approximately 3–4 cells away), and the amplitude of EPSCs was adjusted within approximately 80–150 pA; both of these efforts were made to minimize the potential spatial effect (Williams and Mitchell, 2008). Evoked EPSCs were recorded at a holding potential of −70 mV, and evoked IPSCs were held at −10 mV. Amplitudes of evoked EPSCs and IPSCs were calculated by averaging 30 traces and measuring the peak (1-ms window) compared with the baseline (1-ms window). All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri). Dynorphin peptides, nor-BNI, and U69593 were provided by the Drug Supply Program of the National Institutes of Health National Institute on Drug Abuse.

2.2.4 Data Acquisition, Analysis, and Statistics

One to three cells were obtained from each rat. For experiments involving cocaine/saline administration, at least four rats were used in each treatment group. The numbers of cells (*n*) was used in all statistics. All results are shown as mean ± SEM. Paired *t* test was used for all results involving comparisons of the peak amplitudes of EPSCs or IPSCs before, during, and after (wash-out period) perfusion of pharmacological manipulations.

2.3 RESULTS

2.3.1 Inhibition of Excitatory Synaptic Currents in MSNs by DynA and B

To examine the effects of dynorphins on excitatory synaptic transmission within the NAc, we used whole-cell voltage-clamp techniques in acute brain slices to record evoked EPSCs in NAc MSNs. We first examined the effect of DynA. After establishing a stable baseline of EPSCs, we perfused the brain slice with DynA (1 μmol/L) through the recording bath. Upon application of DynA, the amplitude of EPSCs in NAc MSNs was significantly decreased (amplitude relative to baseline: 70.9 ± 6.6%, $n = 6$; $p < .01$, paired t test) (Figures 1A and 1B). This DynA-induced effect could not be washed out; it persisted after being perfused by DynA-free bath for the rest of the experiment (> 20 min) (relative to baseline: 76.4 ± 12.9%; $p = .01$, vs. baseline, paired t test) (Figures 1A and 1B). Accompanying the DynA-induced decreased in the amplitude, the paired pulse ratio (PPR) (amplitude of the second EPSC peak over the amplitude of the first EPSC peak, 50-msec interpulse interval) of EPSCs was increased (baseline, 1.12 ± .13; DynA, 1.34 ± .14, $n = 6$; $p = .03$, paired t test) (Figures 1A and 1C), suggesting a presynaptic action of DynA. Similar to DynA, bath application of DynB (1 μmol/L) also decreased the peak amplitude of EPSCs (relative to baseline: 58.5 ± 7.2%, $n = 6$; $p < .01$, paired t test) (Figures 1D and 1E) and increased the PPR of EPSCs (baseline, 1.23 ± .08; DynB, 1.58 ± .22, $n = 6$; $p = .04$, paired t test) (Figures 1D and 1F) in NAc MSNs. Similar to DynA (Figure 1B), the effect of DynB was also only partially washed out (relative to baseline: 87.2 ± 9.9%; $p = .23$, vs. baseline, paired t test) (Figure 1E). Thus, DynA and DynB exhibited similar presynaptic effects on EPSCs in NAc MSNs in naive rats.

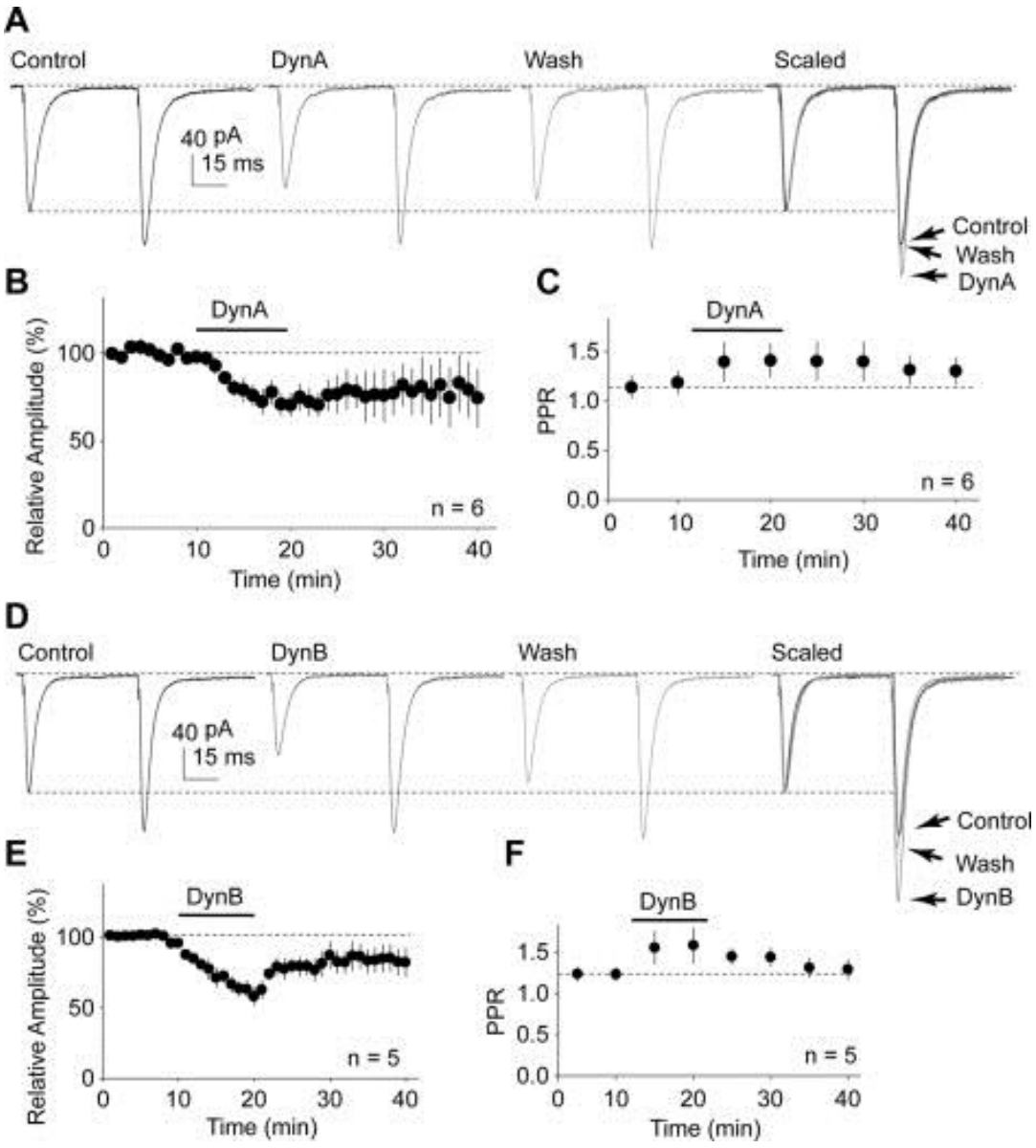

Figure 1. Inhibition of excitatory postsynaptic currents (EPSCs) in nucleus accumbens (NAc) medium spiny neurons (MSNs) by dynorphin A (DynA) and B (DynB).
(A) Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of DynA. The EPSCs were elicited in a paired-pulse manner. **(B)** Summarized results showing that perfusion of DynA inhibited the amplitude of EPSCs in NAc MSNs, and the effect could not be washed. **(C)** Summarized results showing that, accompanying the DynA-mediated inhibition of EPSC amplitude, the paired pulse ratio (PPR) was increased. **(D)** Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of DynB. **(E)** Summarized results showing that perfusion of DynB inhibited the amplitude of EPSCs in NAc MSNs, and the effect could not be washed. **(F)** Summarized results showing that, accompanying the DynB-mediated inhibition of EPSC amplitude, the PPR was increased.

2.3.2 Differential involvements of κ receptors in DynA- and DynB-mediated regulation of EPSCs in NAc MSNs

Opioid κ receptors are enriched in the presynaptic terminals in the dorsal and ventral striatum, and both DynA and DynB exhibit higher affinity for κ receptors over other opioid receptor subtypes (Dhawan et al., 1996, Chen et al., 2007). We thus examined whether the aforementioned effects of DynA were mediated by κ receptors. The approach we used was to test the effects of κ receptor-selective compounds on EPSCs in NAc MSNs. If κ receptors were the key to the observed effects, activation of κ receptors with highly selective agonists should mimic DynA-mediated effects, whereas preventing the activation of κ receptors with highly-selective antagonists should inhibit these effects. U69593 has been demonstrated as a highly selective agonist for κ receptors over other opioid receptors at submicromolar concentrations (Dhawan et al., 1996). In the same experimental setup as in the preceding text, bath application of U69593 (1 μmol/L) decreased the peak amplitude of EPSCs (relative to baseline: 71.7 ± 5.6%, n = 5; p = .02, paired t test) (Figures 2A and 2B) and increased the PPR of EPSCs in NAc MSNs (baseline, 1.20 ± .11; U69593, 1.75 ± .27, n = 5; p = .02, paired t test) (Figures 2A and 2C), an overall effect similar to that of DynA and DynB.

It has been demonstrated that nor-BNI is a highly selective κ receptor antagonist (21). Our results show that, when perfused alone, nor-BNI (0.1 μmol/L) did not significantly affect either the peak amplitude (relative to baseline: 108.3 ± 7.8%, n = 5; p = .45, paired t test) (Figures 2D and 2E) or the PPR (baseline, 1.37 ± .11; nor-BNI, 1.23 ± .06, n = 5, p = .47) (Figures 2D and 2F) of EPSCs in NAc MSNs. Consistent with previous results (Hjelmstad and Fields, 2001, 2003), application of nor-BNI reversed the effects of U69593 on both the peak

amplitude (relative to baseline: U69593, 77.5 ± 4.7%, $p < .01$, vs. baseline; U69593 + nor-BNI, 95.4 ± 8.4%, $p = .67$, vs. baseline; $p = .33$, vs. U69593; $n = 5$) (Figures 2G and 2H) and PPR (baseline, 1.02 ± .11; U69593, 1.66 ± .22, $p = .02$; U69593 + nor-BNI, 1.30 ± .17, $p = .08$; $n = 5$) (Figures 2G and 2I) of EPSCs in NAc MSNs. Thus, if the selectivity of U69593 and nor-BNI holds in the NAc slice as demonstrated in cell lines (Dhawan et al., 1996), our aforementioned results suggest that κ receptor-mediated modulation of EPSCs can be relatively selectively induced by U69593 and reversed by nor-BNI.

With nor-BNI, we examined whether the effects of DynA and DynB on EPSCs in NAc MSNs could be inhibited by inhibition of κ receptors. Our results show that the effects of DynA on both peak amplitude (relative to baseline: DynA, 69.8 ± 4.5%, $p < .01$, vs. baseline; DynA + nor-BNI, 90.2 ± 10.7%, $p = .38$, vs. baseline; $n = 6$) (Figures 2J and 2K) and PPR (baseline, 1.35 ± .11; DynA, 1.68 ± .18, $p = .02$, vs. baseline; DynA + norBNI, 1.44 ± .14, $p = .17$, vs. baseline, $n = 5$) (Figures 2J and 2L) of EPSCs were reversed by application of nor-BNI. In contrast, the effects of DynB on the peak amplitude (relative to baseline: DynB, 60.2 ± 8.7%, $p < .01$, vs. baseline; DynB + nor-BNI, 83.8 ± 5.6%, $p = .02$, vs. baseline; $n = 6$) (Figures 2M and 2N) and PPR (baseline, 1.29 ± .14; DynB, 1.76 ± .24, $p = .02$, vs. baseline; DynB + norBNI, 1.67 ± .10, $p = .02$, vs. baseline; $n = 5$) (Figures 2M and 2O) of EPSCs in NAc MSNs were only partially reversed. These results taken together suggest that the effects of DynA on EPSCs in NAc MSNs are primarily mediated by activation of κ receptors, whereas the effects of DynB are mediated not only by activation of κ receptors but also by κ receptor-independent mechanisms.

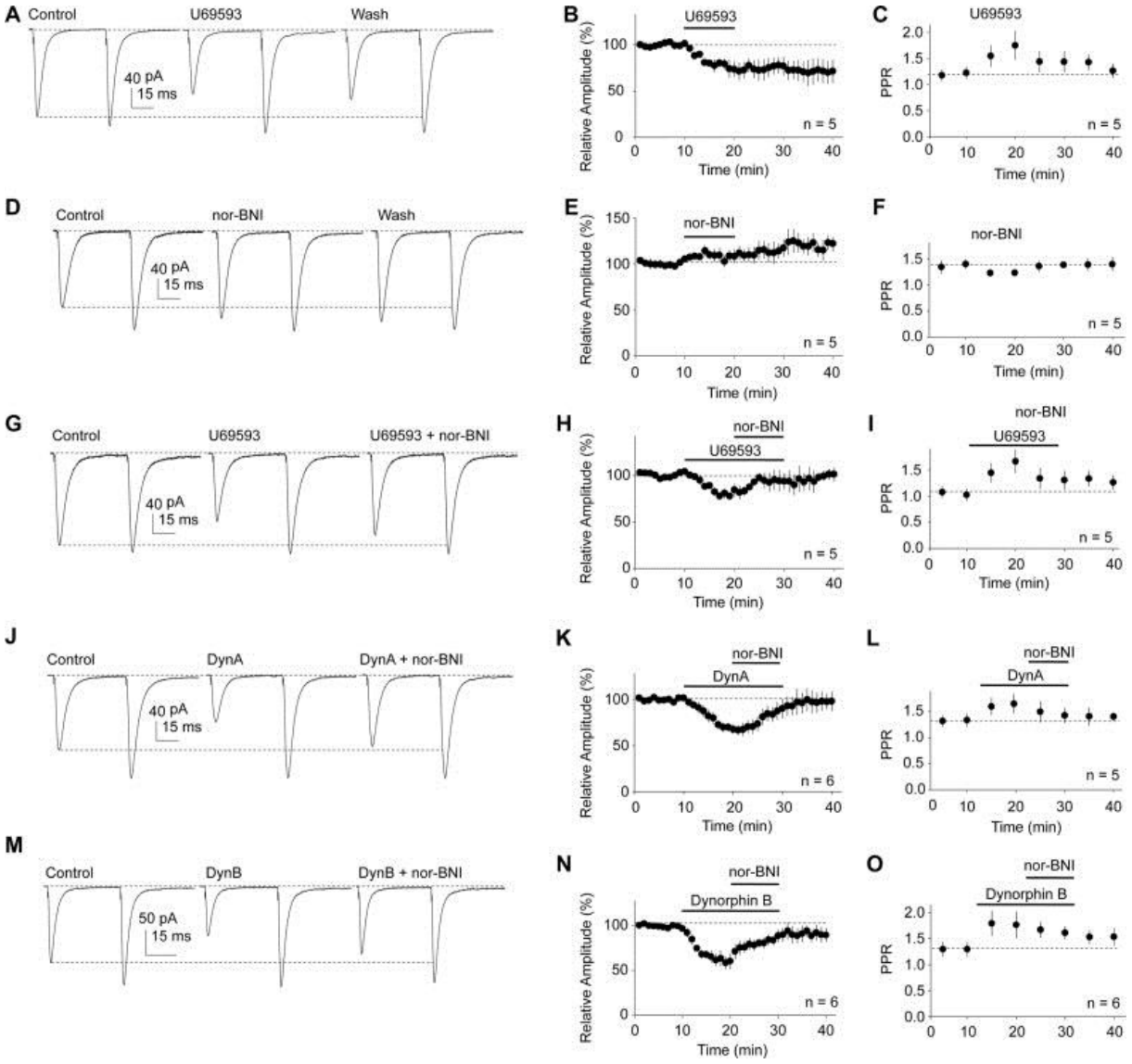

Figure 2. Differential involvements of κ receptors in DynA- and DynB-mediated regulation of EPSCs in NAc MSNs.

(A) Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of U69593. The EPSCs were elicited in a paired-pulse manner. **(B)** Summarized results showing that perfusion of U69593 inhibited the amplitude of EPSCs in NAc MSNs, and the effect could not be washed. **(C)** Summarized results showing that, accompanying the U69593-mediated inhibition of EPSC amplitude, the PPR was increased. **(D)** Examples showing that the amplitude of EPSCs in NAc MSNs was not affected by perfusion of nor-binaltorphimine (nor-BNI). **(E)** Summarized results showing that perfusion of nor-BNI did not significantly affect the amplitude of EPSCs in NAc MSNs. **(F)** Summarized results showing that the PPR of EPSCs was not significantly affected by perfusion of nor-BNI. **(G)** Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of U69593, and this effect was reversed by application of nor-BIN. **(H)** Summarized results showing that U69593-induced inhibition of EPSC amplitude in NAc MSNs could be reversed by application of nor-BNI. **(I)** Summarized results showing that U69593-induced increase in PPR could be reversed by application of nor-

BNI. **(J)** Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of DynA, and this effect was reversed by application of nor-BIN. **(K)** Summarized results showing that DynA-induced inhibition of EPSC amplitude in NAc MSNs could be reversed by application of nor-BNI. **(L)** Summarized results showing that DynA-induced increase in PPR could be reversed by application of nor-BNI. **(M)** Examples showing that the amplitude of EPSCs in NAc MSNs was decreased by perfusion of DynB, and this effect was not completely reversed by application of nor-BNI. **(N)** Summarized results showing that DynB-induced inhibition of EPSC amplitude in NAc MSNs could be partially reversed by application of nor-BNI. **(O)** Summarized results showing that U69593-induced increase in PPR could be partially reversed by application of nor-BNI. Other abbreviations as in Figure 1.

2.3.3 DynA-mediated Regulation of EPSCs in NAc MSNs is Disrupted by Exposure to Cocaine

Exposure to cocaine increases the level of dynorphins in NAc, and this elevated dynorphin tone has been implicated in several NAc-based behavioral alterations (Shippenberg et al., 2007, Shippenberg, 2009). We asked whether DynA/B-mediated modulation of excitatory synaptic transmission in NAc was affected by exposure to cocaine. With the same experimental setup as described in the preceding text, we observed that the effects of DynA on the peak amplitude (relative to baseline: DynA, 116.1 ± 8.2%, n = 7, p = .11) (Figures 3A and 3B) and PPR (baseline, 1.36 ± .12; DynA, 1.16 ± .04, n = 7, p = .36) (Figures 3A and 3C) were abolished in cocaine-pretreated rats (1–2 days withdrawal after 5-day repeated IP injection).

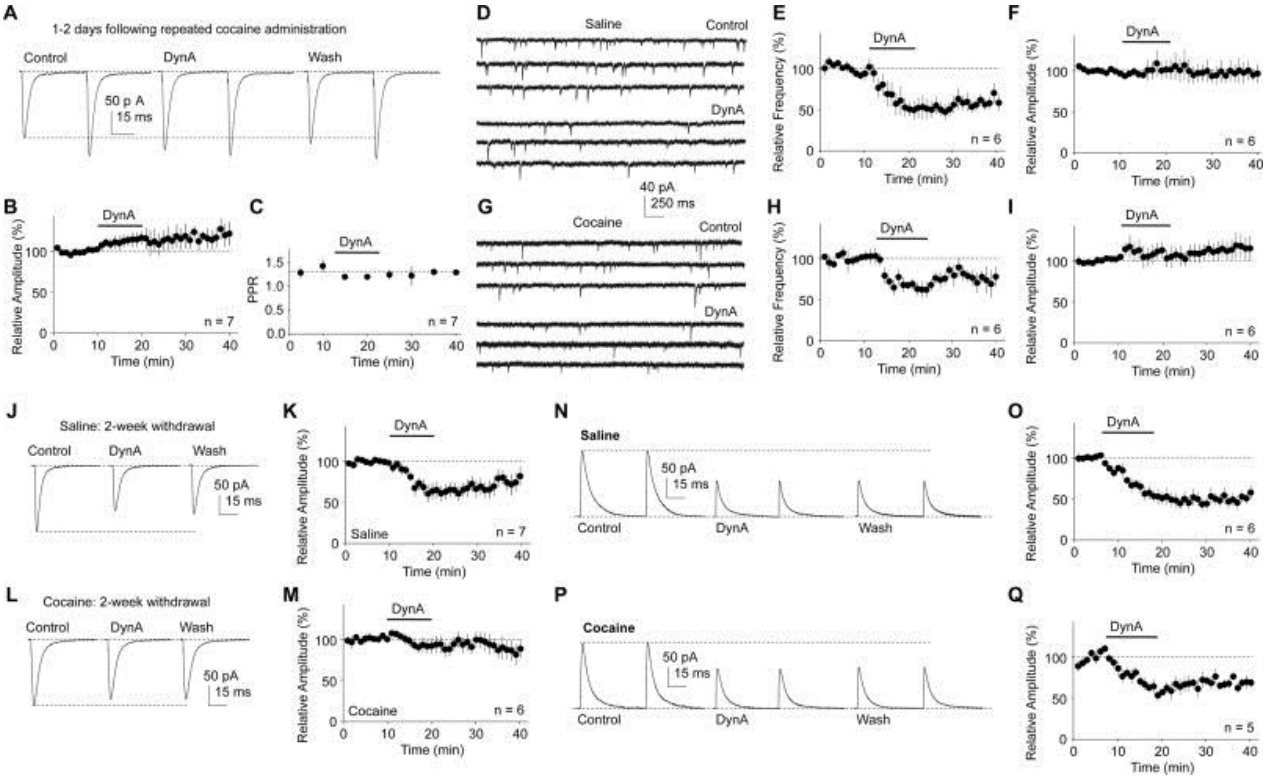

Figure 3. DynA-mediated regulation of EPSCs in NAc MSNs was disrupted by exposure to cocaine.

(A) Examples showing that the amplitude of EPSCs in NAc MSNs was not affected by perfusion of DynA in rats with 1 or 2 days of withdrawal from repeated cocaine administration. The EPSCs were elicited in a paired-pulse manner. **(B)** Summarized results showing that DynA-induced inhibition of EPSC amplitude in NAc MSNs was not present in rats with 1 or 2 days of withdrawal from repeated cocaine administration. **(C)** Summarized results showing that DynA-induced increase in PPR was not present in rats with 1 or 2 days of withdrawal from repeated cocaine administration. **(D–F)** Example traces **(D)** and summarized results **(E**, **F)** showing that the frequency **(E)** but not the amplitude **(F)** of miniature excitatory postsynaptic currents (mEPSCs) in NAc MSNs from saline-treated rats was decreased by perfusion of DynA. **(G–I)** Example traces **(G)** and summarized results **(H**, **I)** showing that the frequency **(H)** but not the amplitude **(I)** of mEPSCs in NAc MSNs from cocaine-treated rats was decreased by perfusion of DynA. **(J, K)** Examples **(J)** and summarized results **(K)** showing that perfusion of DynA significantly decreased the amplitude of evoked EPSCs in rats with 2-week withdrawal from repeated saline treatment. **(L, M)** Examples **(L)** and summarized results **(M)** showing that perfusion of DynA did not affect the amplitude of evoked EPSCs in rats with 2-week withdrawal from repeated cocaine treatment. **(N)** Examples showing inhibitory postsynaptic currents (IPSCs) in NAc MSNs from saline-treated rats (with 1-day withdrawal) before, during, and after perfusion of DynA. **(O)** Summarized results showing that perfusion of DynA significantly inhibited the amplitude of IPSC in NAc MSNs from saline-treated rats. **(P)** Examples showing IPSCs in NAc MSNs from cocaine-treated rats (with 1-day withdrawal) before, during, and after perfusion of DynA. **(Q)** Summarized results showing that DynA-mediated inhibition of IPSC amplitude in NAc MSNs was still present in cocaine-treated rats (with 1-day withdrawal). Other abbreviations as in Figure 1.

As demonstrated in the preceding text (Figure 1), the effect of DynA on excitatory synaptic transmission seemed to be mediated by the regulation of presynaptic glutamate release. As such, we took an additional step to measure the effect of DynA on mEPSCs, another measure for potential presynaptic alteration, in saline- and cocaine-treated (1-day withdrawal from 5-day cocaine administration) rats. In saline-treated rats, perfusion of DynA significantly inhibited the frequency (normalized frequency: baseline, 104 ± 2 %; DynA, 52 ± 11%, $p < .01$, paired *t* test) (Figures 3D and 3E) but not the amplitude (normalized amplitude: baseline, 100 ± 1%; DynA, 102 ± 12%, $n = 6$, $p = .87$, paired *t* test) (Figures 3D and 3F) of mEPSCs in NAc MSNs. These results, taken together with the effect of DynA on PPR of EPSCs (Figure 1), suggest that DynA selectively inhibited presynaptic release of glutamate in NAc MSNs. Surprisingly, the effect of DynA on mEPSCs remained largely intact in NAc MSNs from cocaine-treated rats (normalized frequency: baseline, 104 ± 2%; DynA, 58 ± 9%, $n = 6$, $p < .01$, paired *t* test; normalized amplitude: baseline, 100 ± 1%; DynA, 96 ± 11%, $n = 6$, $p = .77$, paired *t* test) (Figures 3G– 3I). The intact effect of DynA on mEPSCs and the diminished effect on evoked EPSCs, which were concurrently detected within the same set of NAc neurons ($n = 4$, data not shown), taken together might reflect the differential susceptibility of different pools of presynaptic vesicles to cocaine exposure (see Discussion). Note that no difference in baseline amplitude or frequency of mEPSCS was detected between saline- and cocaine-treated rats (data not shown).

Nonetheless, it seemed that DynA-mediated regulation of evoked EPSCs in NAc MSNs was disrupted by cocaine exposure in a long-lasting manner; DynA-mediated regulation of EPSCs in NAc MSNs remained undetectable 2 weeks after repeated cocaine administration (relative EPSC amplitude during DynA perfusion: saline, 64 ± 9%, $n = 7$, $p < .01$, paired *t* test; cocaine, 113 ± 13%, $n = 6$, $p = .32$, paired *t* test) (Figures 3J– 3M). Collectively, the

aforementioned results suggest that exposure to cocaine disrupts DynA-mediated regulation of evoked excitatory synaptic transmission in a long-lasting manner.

The lack of DynA-mediated regulation of EPSCs in cocaine-treated animals might be mediated by two potential mechanisms: 1) a general disruption of the DynA-signaling or alternatively, or 2) a disruption of the coupling between DynA-signaling and excitatory presynaptic machinery while general DynA-signaling remains intact. To explore these mechanisms, we examined potential DynA-mediated modulation of IPSCs in NAc MSNs from saline- and cocaine-treated (1-day withdrawal from 5-day cocaine administration) rats. Perfusion of DynA significantly inhibited the peak amplitude of IPSCs in NAc MSNs from both saline- (relative IPSC amplitude during DynA perfusion: 57 ± 8%, $n = 6$, $p < .01$, paired t test) (Figures 3N and 3O) and cocaine-treated rats (relative IPSC amplitude during DynA perfusion: 64 ± 6%, $n = 5$, $p < .01$, paired t test) (Figures 3P and 3Q). Thus, it seems that excitatory synaptic transmission is selectively targeted by cocaine-induced DynA-mediated dysregulation in the NAc.

2.3.4 DynB-mediated Regulation of EPSCs in NAc MSNs Is Not Disrupted by Exposure to Cocaine

In contrast to the effect of DynA, short-term withdrawal (1 or 2 days) from repeated cocaine administration did not significantly reduce the effects of DynB on evoked EPSCs (relative EPSC amplitude: DynB, 75.9 ± 10.4%, $n = 7$, $p = .03$; PPR: baseline, 1.24 ± .10; DynB, 1.39 ± .02, $n = 7$, $p < .01$) (Figures 4A– 4C). To selectively examine κ receptor-mediated regulation, we tested the effect of U69593. The effects of the selective κ receptor agonist U69593 (1 μmol/L) were

also abolished in cocaine-pretreated rats (relative EPSC amplitude: U69593, 97.7 ± 2.6%, $n = 7$, $p = .45$) (Figures 4D and 4E), similar to that for DynA (Figure 3). These results, taken together with the results related to DynA (Figure 3), suggest that the κ receptor-mediated modulation of evoked excitatory synaptic transmission in NAc was selectively abolished after exposure to cocaine, whereas the κ receptor-independent effects of DynB remain intact. Then, what are the κ receptor-independent mechanisms that mediate the effect of DynB in cocaine-treated animals? One candidate is μ receptors. The μ receptors are also enriched in the NAc, and activation of μ receptors inhibits excitatory synaptic transmission in NAc MSNs with a seemingly similar presynaptic mechanism (Dhawan et al., 1996). Although dynorphins, including DynB, exhibit preferential affinity to κ receptors over μ and other opioid receptors, the selectivity for κ receptors over μ receptors is limited (Corbett et al., 1982). Thus, it is possible that the μ receptor-coupled signaling pathway was not disrupted by cocaine exposure, allowing for DynB-mediated effects. To examine this possibility, we preinhibited μ receptors and then measured the effect of DynB on evoked EPSCs in NAc MSNs from cocaine-treated rats (1-day withdrawal). The μ receptor-selective antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH(2) (CTAP), was perfused to the NAc slice at 1 μmol/L, a concentration at which CTAP completely prevented μ receptor-mediated modulation of EPSCs in the same preparation ($n = 4$, data not shown). In the presence of CTAP, perfusion of DynB still significantly inhibited the peak amplitude of evoked EPSCs in NAc MSNs in cocaine-treated rats (relative EPSC amplitude during perfusion of DynB: 69 ± 11%, $n = 5$, $p < .05$, paired t test) (Figures 4F and 4G). Thus, neither κ nor μ receptors likely mediated the effects of DynB in cocaine-treated animals.

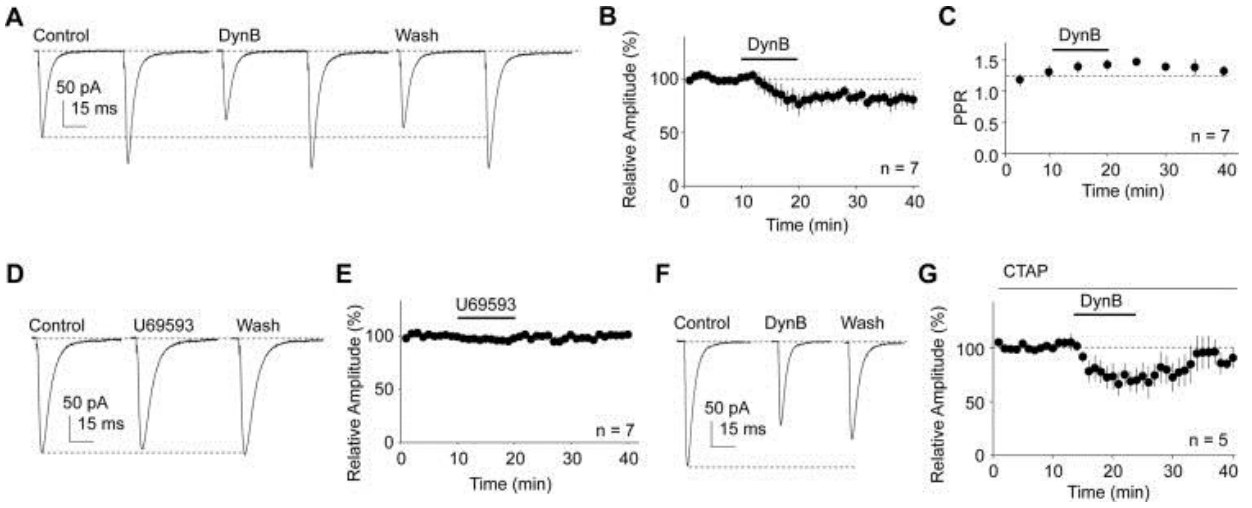

Figure 4. DynB-mediated regulation of EPSCs in NAc MSNs was not disrupted by exposure to cocaine.

(A) Examples showing EPSCs recorded in NAc MSNs from cocaine-treated rats (with 1 or 2 days of withdrawal) before, during, and after perfusion of DynB. **(B)** Summarized results showing that DynB-induced inhibition of EPSC amplitude in NAc MSNs was still present in cocaine-treated rats. **(C)** Summarized results showing that DynB-induced increase in PPR was still present in cocaine-pretreated rats. **(D)** Examples showing EPSCs in NAc MSNs from cocaine-treated rats (with 1 or 2 days of withdrawal) before, during, and after perfusion of U69593. **(E)** Summarized results showing that U69593-induced inhibition of EPSC amplitude in NAc MSNs was not present in cocaine-pretreated rats. **(F)** Examples showing EPSCs in NAc MSNs from cocaine-treated rats (with 1 or 2 days of withdrawal) before, during, and after perfusion of DynB. Recording was made in the presence of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH(2) (CTAP) throughout the experiments. **(G)** Summarized results showing that, in the presence of CTAP, DynB-induced inhibition of EPSC amplitude in NAc MSNs was not present in cocaine-pretreated rats. Other abbreviations as in Figure 1.

2.4 DISCUSSION

Current understanding about dynorphin-signaling-mediated regulation of synaptic transmission within NAc is primarily derived from κ receptor-based studies. In behaving animals, dynorphin peptides are released during behavioral responses, which initiate dynorphin-signaling. Thus, it is important to examine the precise role of dynorphin peptides to depict a complete picture of dynorphin-signaling.

Our present study focused on DynA and DynB, two key dynorphin products from prodynorphin. Our results show that inhibiting κ receptors did not completely prevent DynB-mediated synaptic inhibition (Figure 2), suggesting that—in addition to activation of κ receptors—other mechanisms are also involved in dynorphin-signaling-mediated regulation of synaptic transmission in NAc. These results provide a cellular explanation why different dynorphins exhibit differential psychopharmacological effects (Takemori et al., 1993, Tan-No et al., 2001). Furthermore, DynB is much more abundant than DynA in the NAc (Healy and Meador-Woodruff, 1994). Thus, κ receptor-independent dynorphin-signaling should play a significant role in the NAc upon upregulation of prodynorphins.

An important finding of the present study is that repeated exposure to cocaine abolished the effect of DynA on excitatory but not inhibitory synaptic transmission in NAc. In contrast, the effect of DynB remained largely intact in cocaine-pretreated animals (Figure 4). As suggested by our results, the effect of DynA is primarily mediated by κ receptors. As such, the lack of a DynA effect can be mediated by cocaine-induced alteration in κ receptors or κ receptor-coupled intracellular signaling pathways. For example, if κ receptors are downregulated in the NAc after exposure to cocaine, a diminished effect of DynA would be expected. However, results related to

the effects of cocaine on NAc κ receptors are highly inconsistent; no effect (Schroeder et al., 2003, Bailey et al., 2007), upregulation (Hurd and Herkenham, 1993, Unterwald, 2001, Collins et al., 2002), and downregulation (Rosin et al., 1999, Rosin et al., 2000) have all been reported in cocaine-treated animals. Such discrepant results might reflect the dynamic nature of the NAc κ receptors upon different cocaine procedures. Nonetheless, in addition to up- or downregulation of κ receptors, prestimulation of κ receptor-coupled signaling pathways might also be a contributing factor. It has been consistently shown that the level of dynorphins is increased by exposure to cocaine and other drugs of abuse (Terwilliger et al., 1991, Hurd et al., 1992, Spangler et al., 1993, Cole et al., 1995, Claye et al., 1996). Thus, in the NAc of cocaine-exposed animals, increased dynorphins might strongly and persistently stimulate κ receptors, resulting in either receptor desensitization or a saturation of activation of their coupled signaling pathways. Consequently, an additional application of DynA could not produce further effects. It is important to note that these potential mechanisms seem to only hold for excitatory synaptic transmission, because DynA-mediated modulation of IPSCs in NAc MSNs was largely intact in cocaine-treated animals (Figure 3). This additional result suggests that, mechanistically, cocaine-induced disruption of DynA-signaling is not a global effect. Rather, the DynA-signaling specifically within either the presynaptic glutamatergic terminals or the coupling between DynA-signaling and presynaptic machinery might be primarily targeted by exposure to cocaine, resulting in dysregulation.

Our present data suggest that the inhibitory effects of DynA on excitatory synaptic transmission are achieved by inhibiting the presynaptic release of glutamate (Figure 1). The PPR of evoked EPSCs and the frequency of mEPSCs are two independent measures detecting alterations in presynaptic release of neurotransmitters, and very often a change in one is

accompanied by a change in the other. However, in cocaine-treated rats DynA-induced alterations in both the PPR as well as the amplitude of evoked EPSCs were completely abolished (Figures 3A–3C), whereas the inhibitory effect of DynA on the frequency of mEPSCs remained largely intact (Figures 3D–3I). These seemingly contradicting results were not likely due to technical caveats such as different populations of NAc MSNs, because the lack of effect on the PPR of evoked EPSCs and the presence of effect on the frequency of mEPSCs were also concurrently observed within the same set of neurons (n = 4, data not shown). Indeed, these "mismatched" results might reveal the mechanistic basis for the coupling of DynA-signaling to the release machinery in excitatory presynaptic terminals. It has been demonstrated in hippocampal neurons that evoked EPSCs and mEPSCs are mediated by two different sets of synaptic machineries (Atasoy et al., 2008, Fredj and Burrone, 2009) [but see (Groemer and Klingauf, 2007)]. Particularly for presynaptic release, there are two pools of presynaptic vesicles, one primarily for evoked presynaptic release (e.g., evoked EPSCs), and the other primarily for spontaneous presynaptic release (e.g., mEPSCs) (Fredj and Burrone, 2009). If this holds true for the excitatory synaptic transmission to NAc MSNs, a logical interpretation for the "mismatched" results in evoked EPSCs (Figure 1) and mEPSCs (Figure 3) is that DynA-signaling is coupled to the machineries for both evoked and spontaneous synaptic transmission, and exposure to cocaine selectively disrupts the coupling between the DynA-signaling and evoked synaptic transmission. As such, the mechanism underlying cocaine-induced disruption of DynA-mediated modulation of EPSCs might reside on the molecular and cellular components that selectively govern evoked synaptic transmission, such as activity-dependent calcium influx (and thus calcium channels), calcium sensors (i.e., synaptotagmins), or the "refractory" vesicles (Fredj and Burrone, 2009) that mediate evoked presynaptic release. Nonetheless, given that most biologically functional

synaptic transmissions are "evoked" transmissions, the results related to evoked EPSCs might better reflect the in vivo impact of cocaine administration on DynA-mediated modulation of excitatory synaptic transmission in the NAc.

Our results also suggest that, in contrast to the effect of DynA, the κ receptor-independent component of the DynB-mediated effects on excitatory synaptic transmission in the NAc remain substantially intact in cocaine-exposed rats, albeit the maximal effect is diminished (Figure 4). Thus, the fine balance between κ receptor-dependent and κ receptor-independent modulation of excitatory synaptic transmission might be disrupted by cocaine administration; κ receptor-independent effects might dominate the dynorphin-mediated regulations of excitatory synaptic transmission in the NAc after cocaine exposure.

Taken together, we demonstrate differential effects of dynorphins in the NAc as a function of exposure to drugs of abuse, suggesting the existence of complex cellular behavior of dynorphins in the NAc. The NAc dynorphins significantly contribute to stress and drug withdrawal-induced depressive emotional state, likely through their coupled κ receptor-signaling (Shirayama et al., 2004, Hauser et al., 2005). It has been hypothesized that activation of dynorphin-κ receptors might counteract the development and maintenance of the addictive state (Shippenberg et al., 2007), particularly for drug addiction. The present results provide a more detailed cellular understanding of intra-NAc dynorphins and their potentially differential implications in the development of drug addiction, addiction-associated anhedonic emotional states, and treatment of these emotional and motivational disorders.

3.0 INCREASED EXCITABILITY OF LATERAL HABENULA NEURONS IN ADOLESCENT RATS FOLLOWING COCAINE SELF-ADMINISTRATION[2]

3.1 OVERIEW AND INTRODUCTION

Background: The lateral habenula (LHb) is a brain region that has been critically implicated in modulating negative emotional states and responses to aversive stimuli. Exposure to addictive drugs such as cocaine negatively impacts affective states, an effect persisting longer than acute drug effects. However, the mechanisms of this effect are poorly understood. We hypothesized that drugs of abuse, such as cocaine, may contribute to drug-induced negative affective states by altering the firing properties of LHb neurons, thus changing the signaling patterns from the LHb to downstream circuits.

Methods: Using whole-cell current-clamp recording of acutely prepared brain slices of rats after various periods of withdrawal from cocaine self-administration, we characterized an important heterogeneous subregion of the LHb based on membrane properties.

Results: We found two major relevant neuronal subtypes: burst firing neurons and regular spiking neurons. We also found that LHb regular spiking neurons had higher membrane

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excitability for at least 7 days following cocaine self-administration, likely due to a greater membrane resistance. Both the increase in LHb excitability and membrane resistance returned to baseline when tested after a more prolonged period of 45 days of withdrawal.

Conclusion: This is the first study to look at intrinsic LHb neuron properties following cocaine exposure beyond acute drug effects. These results may help to explain how cocaine and other drugs negatively impact affect states.

Drug addiction involves complex neural circuits and involves an enormous number of cellular and molecular adaptations. Acute exposure to drugs of abuse often elicits an emotional "high," while also inducing negative/aversive effects which out-last the initial positive feelings (Solomon and Corbit, 1974, Koob and Le Moal, 1997). This reduction in affect—one's cognitive emotional state—by increasing negative emotional states and/or reducing positive emotional states may contribute to continued or chronic drug use as the user seeks to alleviate these negative feelings (Solomon and Corbit, 1974, Solomon, 1980, Koob and Le Moal, 1997). Chronic drug use is often a serious condition as maladaptive emotional and motivational states can be developed, leading to compulsive drug use or addiction (Solomon, 1980, Koob and Le Moal, 2008). The opponent process theory posits that these prolonged aversive effects work in opposition to the positive rewarding effects of addictive drugs and that these aversive effects are also one of the major difficulties in abstaining from drug use as users seek drugs in order to mitigate chronic negative affect (Solomon and Corbit, 1974, Solomon, 1980, Koob and Le Moal, 1997, 2001, 2008).

The lateral habenula (LHb) has recently garnered interest for its role in mediating negative rewards and aversive effects (Matsumoto and Hikosaka, 2007) and negative affect

(Yang et al., 2008, Li et al., 2011). LHb neuronal activity is negatively correlated with neuronal activity in positive reward-related regions such as the ventral tegmental area (VTA) (Christoph et al., 1986, Ji and Shepard, 2007, Stamatakis et al., 2013). Additionally, direct application of cocaine to brain slices excites LHb neurons (Good et al., 2013, Zuo et al., 2013), while cocaine exposure has been shown to increase aversive conditioning via the LHb (Jhou et al., 2013).

The LHb is a heterogeneous region and includes several smaller sub-nuclei with physiology, connectivity, and functionality that are currently poorly defined (Andres et al., 1999, Weiss and Veh, 2011, Aizawa et al., 2012). Inputs to the LHb arrive from a variety of brain regions and include TH-positive projections from the VTA, indicating that dopaminergic neurons from the VTA send signals to the LHb (Lecourtier and Kelly, 2007, Hikosaka, 2010, Aizawa et al., 2012, Good et al., 2013). Outputs from the LHb are chiefly glutamatergic and primarily target the VTA and the rostromedial tegmental nucleus (RMTg), which then sends GABAergic signals to the VTA (Ji and Shepard, 2007, Lecourtier et al., 2008, Balcita-Pedicino et al., 2011, Matsui and Williams, 2011, Stamatakis et al., 2013). It appears, then, that the LHb is well-situated to mediate negative affect and aversive behaviors by controlling inhibitory signaling to VTA dopamine neurons via this LHb-to-RMTg-to-VTA pathway (Sesack and Grace, 2010, Balcita-Pedicino et al., 2011).

These circuitry-based reports position the LHb as a critical region for regulating drug-induced negative affect as well. Indeed, there is evidence that cocaine exposure induces synaptic plasticity specifically in the LHb-to-RMTg pathway (Maroteaux and Mameli, 2012), likely leading to increased inhibitory signaling from the RMTg to the VTA. Other studies have also examined the LHb-RMTg-VTA pathway and found supporting evidence for its involvement in cocaine-induced aversive behaviors (Jhou et al., 2013). However, it remains largely unknown

whether exposure to drugs of abuse, such as cocaine, reshape or induce plastic changes within LHb neurons directly. Here, we demonstrate that short-term withdrawal (1-2 days) from cocaine self-administration results in significantly increased intrinsic membrane excitability and membrane resistance of LHb neurons. Given the highly regulated conditions under which LHb neurons operate, this adaptation may significantly increase the response of LHb neurons to incoming signals, thus contributing to increases in LHb signaling to downstream targets such as the RMTg and potentially contributing to the prolonged increases in negative affect following cocaine exposure. The cocaine-induced increase in LHb membrane excitability was maintained for at least 7 days after cocaine self-administration. But, when measured after long-term withdrawal of 45 days, this membrane adaptation had returned to baseline levels. These results indicate that there may be a window of time following exposure to cocaine whereby LHb neurons exhibit cocaine-induced increases in excitability and downstream signaling.

3.2 METHODS

3.2.1 Animals

Upon arrival, male Sprague-Daley Rats (Charles River) weighing 90-110 grams were housed in pairs with 12 h/12 h light/dark cycles and free access to food and water. Animals were allowed to habituate to their cages for at least 6 days before undergoing any procedures. Following catheter surgery, animals were single-housed. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University and were performed in accordance with the guidelines of the National Institutes of Health.

3.2.2 Catheter Surgery

After habituating to their cages, p33-40 rats (weighing between 125 and 150 grams) underwent self-administration catheter surgery. Briefly, rats were anesthetized with a ketamine/xylazine mixture (50–100/5-10 mg/kg, i.p.). A silicone catheter (0.51 inner/0.940 mm outer diameter, HelixMark) was then inserted into the jugular vein and run under the skin to a small incision made between the scapulae where it exited the body and connected to a harness with quick connect luer (SAI infusion technologies) worn by the rat. Throughout the recovery and training period, catheters were flushed daily through the harness with sterile saline solution containing gentamicin (5 mg/mL) and heparin (10 us/mL).

3.2.3 Self-administration Training

Following surgery, the rats were placed back in cages to be single-housed and allowed to recover for 7-12 days before beginning self-administration training. Self-administration operant chambers (Med Associates) contained 2 separate nose-poke holes 6 cm above the grid floor. The harness luer was attached to a swivel with a tether and connected to a syringe loaded into an infusion pump. Nose pokes to the active hole initiated an infusion "reward" of cocaine/saline (0.75mg/kg cocaine or an equivalent volume of saline over 6 s; the volume of each infusion was 90-150 µL and was adjusted based on the body weight of the animal at each training session to meet this criteria), turned off the house light for 20 s, and turned on a separate 6 s cue light. A successful infusion was followed by a 14 s lockout period. During this lockout period, active nose pokes continued to be recorded but failed to initiate any cues or further infusions. At the end of this lockout period, the house light would turn back on, signaling that the active nose poke

hole could now initiate another infusion. Nose pokes in the inactive hole were recorded but elicited no effects. All tests were done using an FR1 schedule.

Rats were randomly divided into cocaine and saline groups for across the 3 chosen withdrawal time points and were placed in the operant chambers for one over-night training session (~12 hours) 8-10 days after surgery. Rats in a cocaine group that failed to receive greater than 30 cocaine infusions during the overnight session were excluded from further testing (~10% of the group). ~24 hours later, rats began a series of daily 2-hour training sessions over 5 consecutive days. Rats in a cocaine group which did not show the ability to distinguish between active and inactive nose pokes or did not receive at least 15 cocaine infusions per 2 hour session were excluded from further study (~10% of the group). At the conclusion of the training, rats from all groups were placed back into their home cages for a 24-48 hour, 5-7 day, or a 43-47 day (referred to as 45 days) withdrawal period before being taken for brain slice preparation.

3.2.4 Brain Slice Preparation

Following the withdrawal period, rats were quickly anesthetized with ~3-4 mL of 99.9% isofluorane in a closed 20x16x16 cm chamber and were decapitated so that the brain could be extracted. Coronal brain slices (260 µm thick) containing the LHb were prepared using a VT1200S microtome (Leica). Slices were cut in the presence of 4°C cutting solution containing (in mM): 135 *N*-methyl-D glucamine, 1 KCl, 1.2 KH_2PO_4, 0.5 $CaCl_2$, 1.5 $MgCl_2$, 20 choline-HCO_3, and 11 glucose, saturated with 95% O_2/5% CO_2, with the pH adjusted to 7.4 using HCl, at 300-310 mOsm. After being cut, slices were placed in an incubation chamber in artificial cerebrospinal fluid (aCSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, at 290-294 mOsm, saturated with 95%

O2/5% CO_2 to maintain a pH of 7.4 at 37°C for 30 min. Slices were then allowed to recover for at least 30 min at room temperature before being used for experimentation.

3.2.5 Electrophysiological Recordings

Whole-cell current-clamp recordings were made in the LHb, specifically in the parvocellular and central parts of the medial division of LHb (see Fig. 2a). During recordings, slices were superfused with aCSF that was heated to 31–33°C by passing the solution through a feedback controlled in-line heater (Warner Instruments) before entering the recording chamber. Recordings were made under visual guidance (40x, differential interference contrast optics) with micropipettes (2.5–5 MΩ) filled with a potassium-based internal solution containing (in mM): 130 $KMeSO_3$, 10 KCl, 0.4 EGTA, 10 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP, and 2 $MgCl_2$-$6H_2O$, pH 7.3, 294 mOsm.

The holding current for each cell was adjusted such that the cell maintained a membrane potential of -65 mV, which is close to the average resting membrane potential of these neurons (Wilcox et al., 1988). Though rare, cells requiring currents greater than +/-20 pA to reach this holding potential were excluded for data collection. Series resistance was 9 – 20 MΩ, uncompensated, and monitored continuously during recording. Recordings with a change in series resistance greater than 20% for the duration of data collection were not accepted for data analysis. To record evoked action potential firing, current injection steps were generated using Clampex software (Molecular Devices). The range of -50 to +90 pA, 10 pA increments/steps, and 0.1 Hz/step was chosen based on calibration trials to find appropriate current steps which would elicit the best range of action potential spikes across the full sample of recorded LHb neurons. Voltage traces were recorded with a MultiClamp 700B amplifier, filtered at 3 kHz,

amplified 5 times, and then digitized at 20 kHz. 3 consecutive series of 10 pA steps (from -50 to +90 pA) were recorded from each cell. Cells were allowed ~5 minutes to stabilize after achieving the whole-cell patch configuration before data collection began.

3.2.6 Drugs and Reagents

Cocaine-HCl was supplied by the Drug Supply Program of the National Institute of Drug Abuse. All other chemicals were purchased from Sigma-Aldrich.

3.2.7 Statistics and Data Analysis

All cell measurements were averaged across 3 consecutive trials. The number of action potentials evoked by each current injection step was used as a measure of cell membrane excitability. If the number of peaks at any current step varied more than 20% across any of the 3 trials, the cell was excluded from analysis for being unstable. Membrane resistance measurements for each cell were calculated using Ohm's law by taking the difference in the cell's voltage between the final 100 ms of each negative current step compared to the cell's baseline and dividing by the amount of current injected. The threshold for action potentials was measured as the point at which the voltage level of the cell slopes upwards at >25 mV/ms during the final rise to form an action potential peak. Using this threshold as a baseline, the fast-decaying afterhyperpolarization (fAHP) was measured as the lowest point 2-5 ms after the peak of the action potential and the medium-duration afterhyperpolarization (mAHP) was measured as the lowest point 20-40 ms after the peak of the action potential. Neurons which did not demonstrate either a clear fAHP or mAHP phase were excluded from analysis of that phase. All measurements were taken from the

same set of recorded cells with none being excluded, unless specifically stated. Student's t-test was used to compare single data points, while two-way repeated measures ANOVA was used to compare treatment groups across multiple data points (spike numbers across all current steps and treatment groups, or behavioral data across multiple days and treatment groups). Results are shown as mean +/- SEM.

3.3 RESULTS

3.3.1 Self-administration of Cocaine or Saline

To test the effects of cocaine exposure on LHb neurons, a self-administration training model was used. Rats received 5-day self-administration training after an initial over-night training session. Rats were trained to nose-poke for 0.75 mg/kg infusions of cocaine/saline during 2 hour sessions. Animals in short-term (ST), moderate-term (MT), and long-term (LT) withdrawal groups received identical training and access to cocaine/saline. ANOVA was used to compare the number of rewards received across all treatment groups and training days, whereby rewards infusions was the dependent variable and the training day and treatment group were fixed factors. As expected, comparisons between the ST, MT, and LT withdrawal groups for rats revealed no differences between reward infusions after a Bonferroni posttest (ST vs. LT: saline, $p = 1.00$, $n = 6, 3$; cocaine, $p = 1.00$, $n = 6, 3$; ST vs. MT: saline, $p = 1.00$, $n = 6, 4$; cocaine, $p = 1.00$, $n = 6, 6$; MT vs. LT: saline, $p = 1.00$, $n = 4, 3$; cocaine, 1.00, $n = 6, 3$). Thus, the behavioral results were combined across withdrawal groups. Analysis comparing saline and cocaine treatment groups revealed that rats in combined cocaine treatment groups nose poked for more

infusions than rats in the combined saline group (**Fig. 5**; $p < 0.0001$, $F[1, 139] = 562.64$, n coc/sal = 15/13). The individual day of training had no effect ($p = 0.69$, $F[4, 139] = 0.56$, n coc/sal = 15/13) and no interaction effects were present across withdrawal groups or training days. These results confirm that this 5-day self-administration procedure is sufficient to both initiate and measure cocaine-seeking behavior in adolescent rats and that these rats demonstrate equivalent cocaine-seeking across withdrawal groups.

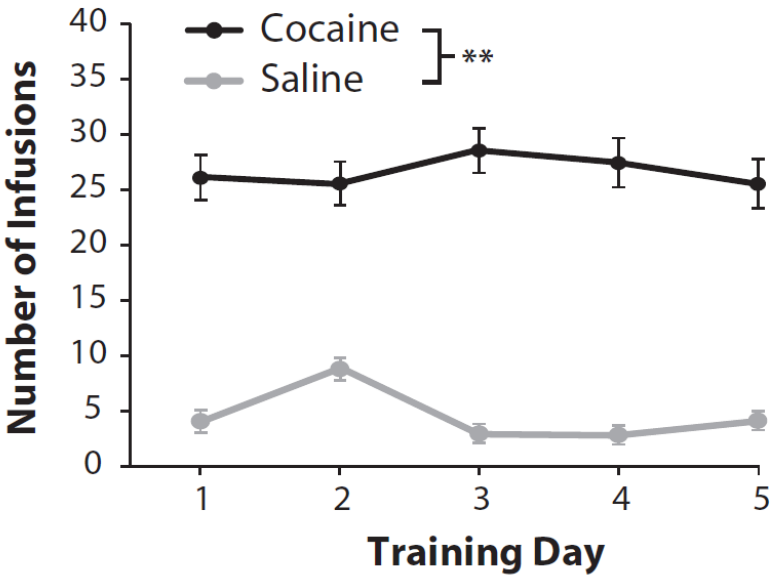

Figure 5. Self-administration leads to cocaine-seeking behavior in rats.
Rats receiving cocaine nose-poked for more infusions than rats receiving saline. The graph shows the average number of infusions for rats in cocaine groups and saline groups across the 5 daily 2-hour self-administration training sessions. Data from ST, MT, and LT withdrawal groups were combined (n of animals per treatment condition: saline ST, 6; MT, 4; LT, 3; total = 13; cocaine ST, 6; MT, 6; LT, 3; total = 15).

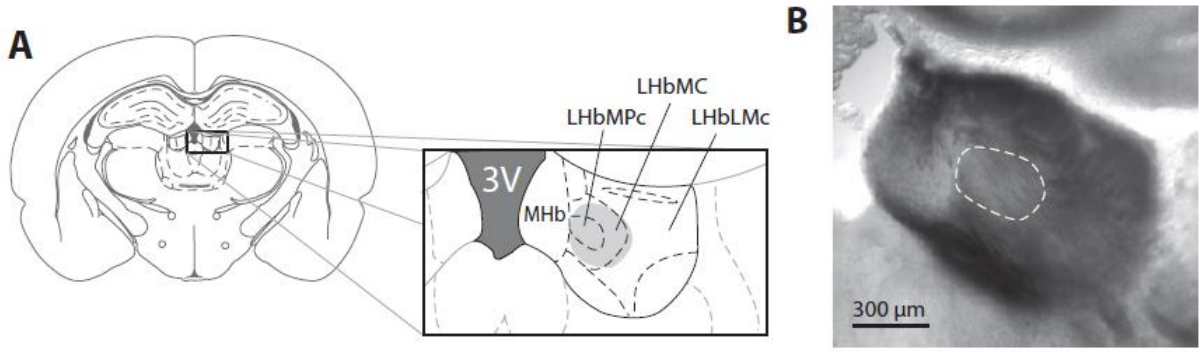

Figure 6. The LHb and subnuclei regions.
(*a*) Diagram showing the parvocellular and central regions of the medial division of the LHb where cells were recorded (shaded region). 3V, third ventricle; LHbMPc, parvocellular part of the medial division of the lateral habenula; LHbMC, central part of the medial division of the lateral habenula; LHbLMC, magnocellular part of the lateral division of the lateral habenula. (*b*) Differential interference contrast image of the habenula of a rat brain slice. The LHb region of interest is apparent from natural markings in the surrounding areas and is outlined by a dashed white line in this picture.

3.3.2 Characterization of Two LHb Neuron Subtypes

24 to 48 hours following the fifth and final self-administration training session, rats were sacrificed to obtain coronal brain slices containing the LHb for whole-cell current-clamp recordings. Cells located in the parvocellular or central parts of the medial division of the lateral habenula were preferentially targeted for recording (simply referred to as LHb hereafter, **Fig. 6*a***), as cells in these regions have been shown to receive the highest density of incoming TH-positive fibers originating from the VTA (Aizawa et al., 2012, Good et al., 2013). The target region was easily discernable under standard differential interference contrast optics (**Fig. 6*b***).

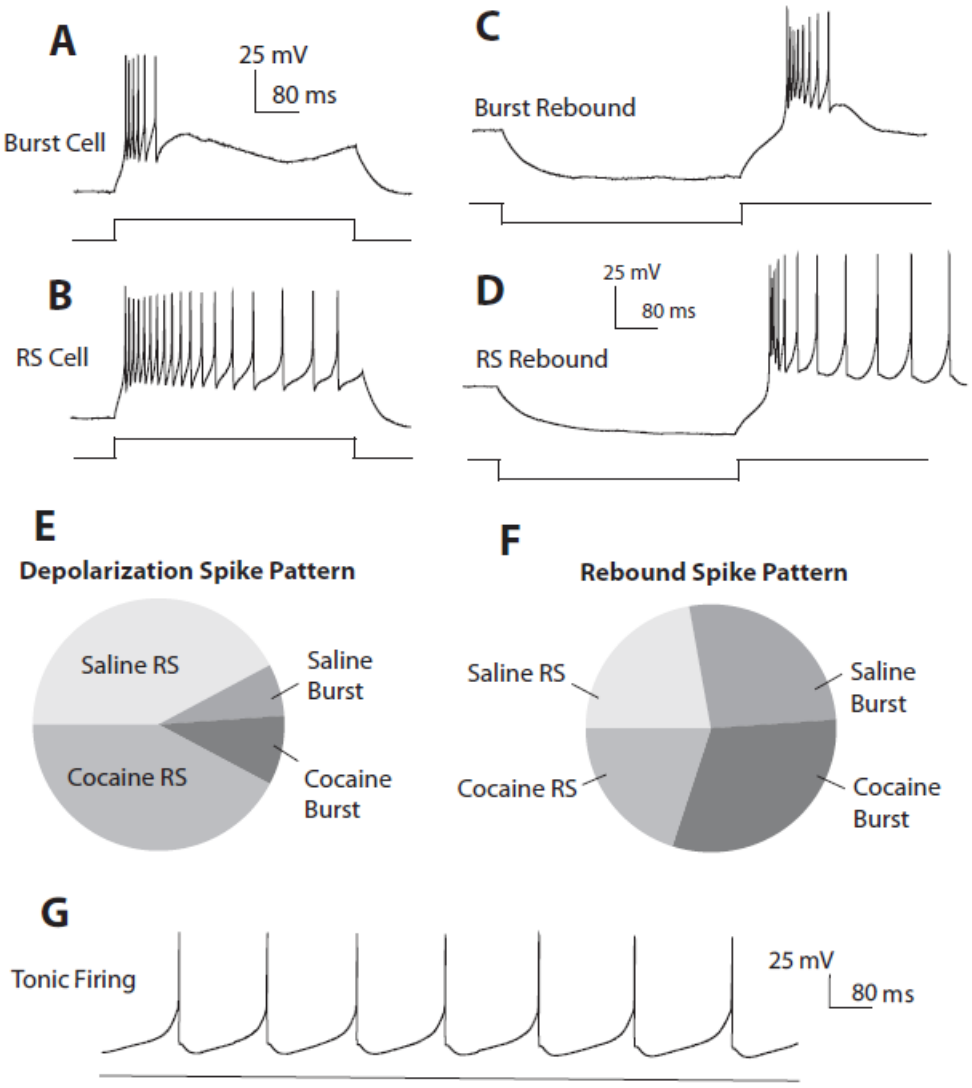

Figure 7. Cell types and spiking patterns in the LHb.
(*a*) Example traces showing a typical burst firing (BF) pattern and (*b*) regular spiking (RS) pattern following depolarizing injections of current. (*c*, *d*) Example traces showing typical (*c*) BF and (*d*) RS rebound spiking patterns. (*e*) Chart showing the relative ratios of BF and RS spiking LHb cells during depolarization following saline (BF = 3, RS = 19, total = 22; 6 rats) or cocaine (BF = 4, RS = 19, total = 23; 6 rats) self-administration training. (*f*) Chart showing the relative ratios of BF and RS rebound spiking patterns in LHb cells following saline (BF = 12, RS = 10, total = 22; 6 rats) or cocaine (BF = 14, RS = 9, total = 23; 6 rats) self-administration training. (*g*) Example trace showing a typical ~5 Hz tonic firing pattern from an LHb cell at rest.

A survey of LHb cells revealed 2 relevant major neuronal types: burst firing (BF) cells and regular spiking (RS) cells (**Fig. 7*a, b***). Continued observation revealed that 3 of 22 recorded cells from saline-treated animals showed clear BF characteristics, while 4 of 23 recorded cells from cocaine-treated animals showed clear BF characteristics (**Fig. 7*e***), indicating that cocaine exposure does not shift the population composition of BF/RS cell types in the LHb.

All recorded LHb neurons also exhibited action potentials evoked by releasing a hyperpolarizing stimulation, termed rebound spiking (Wilcox et al., 1988, Chang and Kim, 2004, Weiss and Veh, 2011) (**Fig. 7*c, d***). Similar to depolarization spiking in LHb neurons, rebound spiking occurs in trains of RS or BF patterns. These rebound spike trains continue for various extended periods of time depending upon the magnitude of the preceding hyperpolarization. Interestingly, the rebound spiking pattern did not necessarily match the depolarization spiking pattern within the same cell, even at the same holding potential. Whereas only 3 of 22 cells recorded from saline treated animals exhibited BF spike patterns during depolarization, 12 of the 22 recorded cells showed BF spike patterns following hyperpolarization (**Fig. 7*e, f***). Similarly, while only 4 of 23 recorded cells from cocaine-treated animals had BF spike patterns during depolarization, 14 of these 23 cells had BF rebound spike patterns (**Fig. 7*e, f***). All cells which showed BF spiking during depolarization also showed BF rebound spiking. The similarity of these sample numbers between the saline and cocaine groups, again, indicates that cocaine exposure does not shift the population composition or resting potential of cell types within the LHb.

Upon achieving a whole-cell patch-clamp configuration, most LHb neurons demonstrated a variety of tonic firing patterns, ranging between 0.1 and 10 Hz (**Fig. 7*g***). This tonic firing was variable and unstable over time though, and even disappeared in many cases ~10-20 minutes

after cells came to rest. Though, continued persistence or even intensification in other cases complicated certain measurements (such as rheobase current). Recorded neurons were injected with current (up to +/-20 pA) in order to maintain a resting potential near -65 mV. A majority (~85%) of LHb neurons demonstrated RS spiking patterns, while a minority (~15%) demonstrated BF spiking patterns. It should also be noted that a relatively small amount of current is needed to bring these LHb neurons to their action potential threshold. The current steps used in the present study ranged from -50 to +90 pA, where spike numbers began to plateau in some cells. A majority of recorded LHb neurons fire multiple spikes with just a 400 ms injection of +10 pA, confirming that neurons in the LHb are highly regulated and sensitive to incoming signals (Wilcox et al., 1988, Chang and Kim, 2004, Weiss and Veh, 2011).

3.3.3 Membrane excitability of LHb neurons is increased after short-term withdrawal from cocaine self-administration

To examine the impact of cocaine self-administration on the membrane excitability of LHb cells, we elicited action potentials from these neurons using a series of current injection steps (-50 to +90 pA, 10 pA increments) 24-48 hours after the final self-administration training session. Cells that demonstrated BF spiking during depolarization (~15% of cells) were excluded from excitability analysis due to their irregular firing pattern. Spike numbers at each current step were counted as a measure of the membrane excitability (**Fig. 8*a***). Two-way repeated measures ANOVA with spike number as the dependent variable repeated at each current step for both saline and cocaine treatment groups revealed that animals from the treatment group had a

significant effect on the membrane excitability in LHb cells 24-48 hours after the final cocaine exposure when compared to saline-exposed controls (**Fig. 8*b***, $p < 0.05$, $F[1, 36] = 5.62$, $n = 38$). Bonferroni's multiple comparisons test was performed to detect significant differences between treatment groups at each current step.

We then looked to examine additional properties of these LHb neurons in an attempt to determine contributing factors to the observed cocaine-induced increase in the membrane excitability. We first measured the threshold of action potentials in single-standing spikes. There was no significant differences in the threshold for action potentials between saline and cocaine exposed rats (**Fig. 8*c***, $p = 0.48$, n sal/coc = 19/19). We next calculated the membrane resistance of the LHb cells by measuring the cell's change in potential in response to negative current injections (-10 to -50 pA). We found that the membrane resistance was increased in cocaine-treated rats compared to saline-treated rats (**Fig. 8*d***, $p < 0.05$, n sal/coc = 19/19). We also measured fast and medium components of AHPs (**Fig. 8*e***) and observed no differences in these two parameters between cocaine and saline treated groups (**Fig. 8*f***; fAHP, $p = 0.91$, n sal/coc = 13/16; mAHP, $p = 0.49$, n sal/coc = 9/6). Thus, an increase in membrane excitability is correlated with an increase in membrane resistance.

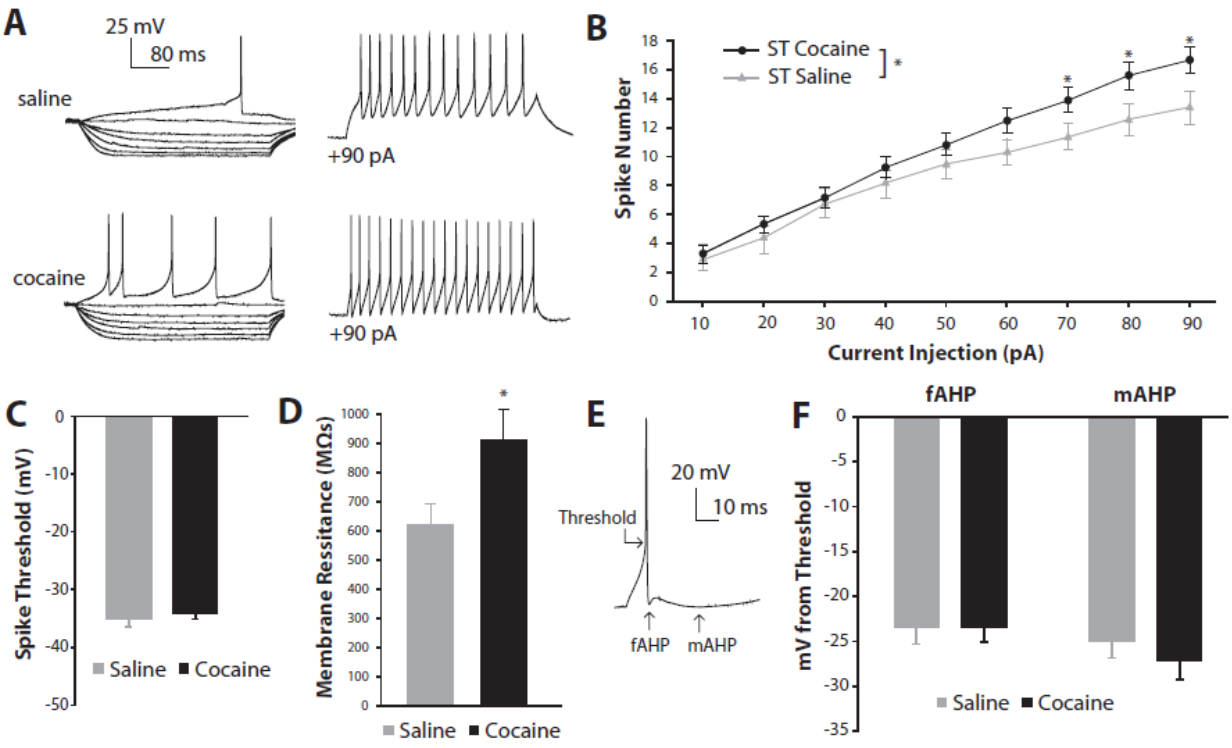

Figure 8. LHb neuron characteristics 1-2 days after cocaine/saline self-administration.
(*a*) Example traces showing typical current steps from -50 to +10 pA (left) and +90 pA (right) in LHb neurons after saline (top) or cocaine (bottom) self-administration. (*b*) Plot showing the mean number of spikes fired at each current step from LHb neurons 24-48 hours after cocaine or saline self-administration training (saline/cocaine, n = 19/19; rats = 6/6). (*c*) Graph showing the mean threshold of action potentials (saline/cocaine, n = 19/19; rats = 6/6). (*d*) Graph showing the mean input resistance of LHb cells (saline/cocaine, n = 19/19; rats = 6/6). (*e*) Example of fAHP and mAHP measurement locations on a typical isolated spike trace. (*f*) Graph of mean fAHP (saline/cocaine, n = 10/8; rats = 6/6) and mAHP (saline/cocaine, n = 13/15; rats = 6/4) measurements relative to spike threshold. *, $p < 0.05$ based on ANOVA comparison in (*b*) and Ttest in (*d*).

3.3.4 Membrane excitability of LHb neurons following long-term withdrawal from cocaine

After observing that cocaine self-administration leads to an increase in the membrane excitability of LHb neurons 24-48 hours later, we then looked at a more protracted withdrawal time point at 45 days to determine if these changes were persistent. The same cell characteristics measured at 24-48 hours of withdrawal were then measured after 45 days of withdrawal. Again, spike numbers at each current step were counted as a measure of the membrane excitability (**Fig. 9*a***). Two-way repeated measures ANOVA using spike number as the dependent variable repeated at each current step for cocaine LT and saline LT treatment groups revealed no significant effect of the treatment on the number of spikes across all current steps ($F[1, 23] = 0.17$, $n = 25$, $p = 0.68$). Thus, it appears that the initial cocaine-induced increase in membrane excitability returns to baseline levels at some time point after 48 hours of withdrawal (**Fig. 9*b***). Additionally, the membrane resistance no longer differed significantly from saline-exposed controls 45 days after the last cocaine exposure (**Fig. 9*d***). All other cellular measures including action potential threshold, fAHP, and mAHP also showed no statistical differences between treatment groups after LT withdrawal (**Fig. 9*c, e, f***). Taken together, these results indicate that the cocaine-induced increase in LHb cell excitability returns to baseline at some point between 2 and 45 days of withdrawal from cocaine and is correlated with changes in membrane resistance.

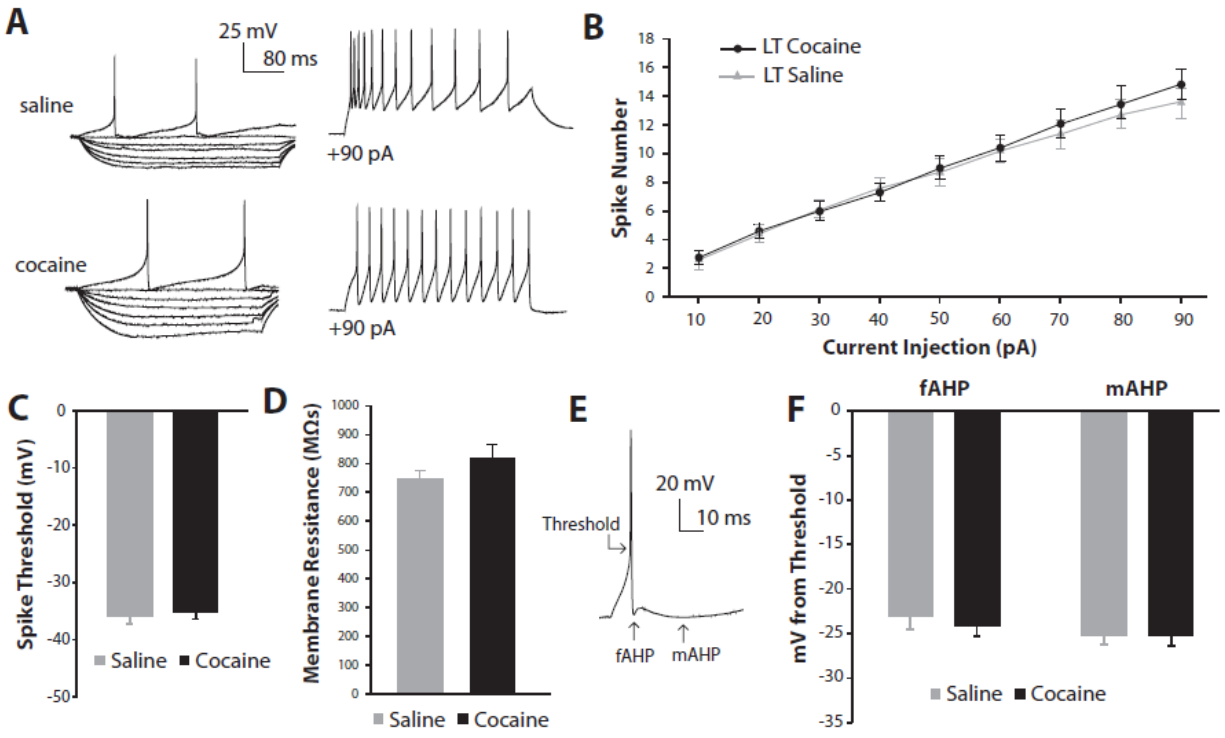

Figure 9. LHb neuron characteristics 45 days after cocaine/saline self-administration.
(*a*) Example traces showing typical current steps from -50 to +10 pA (left) and +90 pA (right) in LHb neurons after 45 days of withdrawal from saline (top) or cocaine (bottom) self-administration. (*b*) Plot showing the mean number of spikes fired at each current step from LHb neurons 45 days after cocaine or saline self-administration training (saline/cocaine, n = 10/15; rats = 3/3). (*c*) Graph showing the mean threshold of action potentials (saline/cocaine, n = 10/15; rats = 3/3). (*d*) Graph showing the mean membrane resistance of LHb cells (saline/cocaine, n = 10/15; rats = 3/3). (*e*) Example of fAHP and mAHP measurement locations on a typical isolated spike trace. (*f*) Graph of mean fAHP (saline/cocaine, n = 6/10; rats = 3/3) and mAHP (saline/cocaine, n = 7/12; rats = 3/3) measurements relative to spike threshold.

3.3.5 Membrane excitability of LHb neurons following moderate-term withdrawal from cocaine

After observing that the cocaine-induced increases in membrane excitability and membrane resistance returned to saline-control levels by day 45 of withdrawal, we decided to examine a 5-7 day moderate term (MT) withdrawal point to better-understand the time course of these observed changes. Again, the same cell characteristics were measured at this MT time point as were measured at the ST and LT withdrawal time points. A two-way repeated measures ANOVA using spike number as the dependent variable repeated at each current step for saline MT and cocaine MT treatment groups showed that the cocaine MT treatment had a significant increase in cell excitability compared to the cocaine ST group ($F[1, 35] = 5.39$, $n = 37$, $p < 0.05$), similar to the cocaine ST treatment group (**Fig. 10*a, b***). Bonferroni's multiple comparisons test was used to check for differences at each current step. This result reveals that LHb neurons maintain increased levels of excitability until at least 7 days after cocaine self-administration training (a Bonferroni posttest comparing withdrawal days 5-7 showed no differences between withdrawal days, $p = 1.00$ for all comparisons). Measurements of other cellular characteristics including the action potential threshold, fAHP, and mAHP again revealed no significant effects of cocaine at the MT withdrawal point (**Fig. 10*c, e, f***). However, the membrane resistance of LHb cells after MT withdrawal from cocaine self-administration was again significantly different from saline controls (**Fig. 10*d***, $p < 0.01$, n sal/coc = 18/19), further supporting a correlation between membrane excitability and membrane resistance.

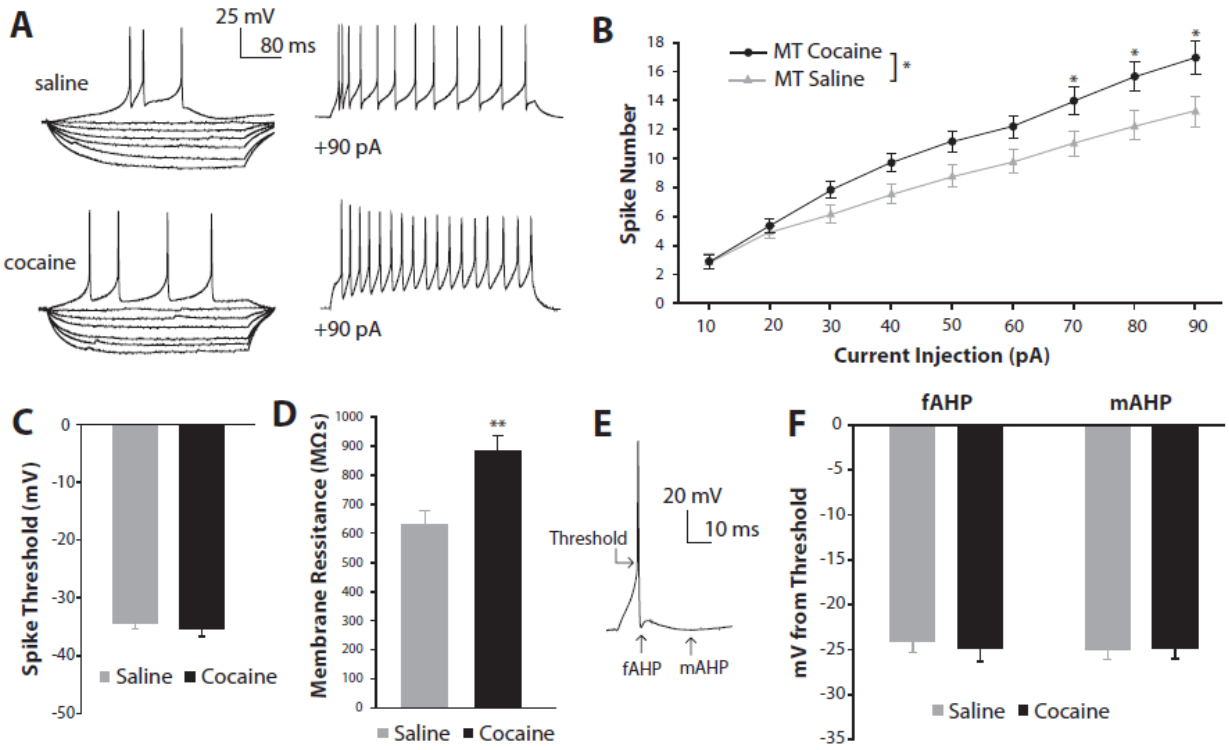

Figure 10. LHb neuron characteristics 5-7 days after cocaine/saline self-administration. (*a*) Example traces showing typical current steps from -50 to +10 pA (left) and +90 pA (right) in LHb neurons after 5-7 days of withdrawal from saline (top) or cocaine (bottom) self-administration. (*b*) Plot showing the mean number of spikes fired at each current step from LHb neurons 5-7 days after cocaine or saline self-administration training (saline/cocaine, n = 18/9; rats = 4/6). (*c*) Graph showing the mean threshold of action potentials (saline/cocaine, n = 18/19; rats = 4/6). (*d*) Graph showing the mean membrane resistance of LHb cells (saline/cocaine, n = 18/19; rats = 4/6). (*e*) Example of threshold, fAHP, and mAHP measurement locations on a typical isolated spike trace. (*f*) Graph of mean fAHP (saline/cocaine, n = 15/13; rats = 4/6) and mAHP (saline/cocaine, n = 14/14; rats = 4/6) measurements relative to spike threshold. *, $p < 0.05$, based on ANOVA comparison; **, $p < 0.01$ based on Ttest.

3.4 DISCUSSION

The present study demonstrates that LHb RS neurons have increased membrane excitability 24-48 hours following self-administration of cocaine with lasts until at least 7 days after the last cocaine exposure. There was no difference in the distribution of cell types, action potential threshold, or fast/medium duration hyperpolarization potentials within the LHb after cocaine self-administration. As an increase in membrane resistance was observed in correlation with the increase in excitability of LHb cells after ST and MT withdrawal, it seems likely that the observed increase in cell excitability is at least partially mediated by an increase in membrane resistance. This cocaine-induced increase in intrinsic excitability amplifies LHb neuron signal transmission. Upon receiving equivalent input, LHb neurons in cocaine-trained animals transmit a greater number of signals to downstream targets, enhancing the contribution of the LHb to the involved circuits. As the LHb appears to mediate negative affect and aversive behaviors, this amplification of LHb signaling may represent a drug-induced alteration contributing to the increased opponent processes and prolonged negative affect known to occur after cocaine and addictive drug use.

Early studies examining the functional and behavioral role of the LHb largely targeted the entire structure, treating it as a homogenous region. However, molecular and ultra-structural characterizations have revealed that the LHb is highly heterogeneous, with many potentially distinct subnuclei involved in specific circuits and functions (Andres et al., 1999, Weiss and Veh, 2011, Aizawa et al., 2012). The present study recorded from neurons located in the parvocellular and central subregions of the medial LHb, which receive the majority of TH-positive projections from the VTA (Aizawa et al., 2012, Good et al., 2013). LHb neurons which receive TH-positive fibers from the VTA primarily send glutamatergic projections to the RMTg (Balcita-Pedicino et

al., 2011, Stamatakis et al., 2013). The receiving neurons in the RMTg then send inhibitory projections to VTA dopamine neurons (Christoph et al., 1986, Jhou et al., 2009, Omelchenko et al., 2009, Stamatakis et al., 2013). Thus, if signals coming out of the LHb are tonically amplified after cocaine exposure, VTA dopamine neurons receive a greater amount of tonic inhibition from the RMTg, causing less dopamine to be released in reward-related regions and reducing positive affect levels of an individual. This scenario supports the hypothesis that increased LHb neuron membrane excitability may contribute to the chronic increase in negative affect states following cocaine and addictive drug exposure.

Increased excitability is observed at least 7 days after the final cocaine exposure, at a time when all acute cocaine effects have subsided. This observed change therefore represents an enduring adaptation in the LHb, as it persists well beyond acute pharmacological effects of cocaine. However, the increase in LHb neuron excitability was not observed at a much later withdrawal time point of 45 days, indicating that it is not a permanent change. Thus, cocaine-induced adaptations in LHb neurons and any posited impact on negative affect appear to be reversible following a short-access cocaine regimen (2 hours per day for 5 days). Hence, there seems to be a window of time following cocaine exposure when LHb transmission is amplified. This window of amplified LHb signaling may be sufficient to trigger additional circuitry changes downstream, such as those found at LHb-to-RMTg synapses after cocaine exposure (Maroteaux and Mameli, 2012), particularly when considering the highly regulated and sensitive nature of LHb neurons (Wilcox et al., 1988, Weiss and Veh, 2011). The cocaine regimen used in this study was relatively mild, and a longer or stronger cocaine treatment regimen may result in larger or longer-lasting cellular effects relative to the results observed here.

Additional analysis was done to further examine the cause of the increased excitability in LHb neurons after cocaine self-administration. A number of membrane properties were measured, including the fast and medium components of AHP, the threshold of action potentials, and the membrane resistance of recorded cells from saline or cocaine treated animals. No differences were found between any of the examined cell characteristics, save for the membrane resistance in the cocaine ST and MT withdrawal group (See **Table 1** and **Fig. 8*d, 6d***). Collectively, these results suggest that cocaine self-administration does not affect sodium channels or big/small conductance calcium-activated potassium channels of LHb cells, and that these channels are not responsible for the increase in excitability. However, membrane resistance does appear to be correlated with an increase in excitability, and can be affected by several factors—resting potassium channels being the principle mediators. Thus, if cocaine exposure were to increase dopamine signaling to LHb neurons by blocking re-uptake, increased dopamine activity at LHb neurons (via D2 and D4 receptors) could hypothetically result in intracellular signaling cascades leading to a reduction in the number of passive potassium channels at the membrane that could persist for days or weeks before returning to baseline levels. There are other potential factors which could lead to increases in membrane excitability, but further investigation into the identities of the specific channels or receptors which might be involved was beyond the scope of the present study. We therefore cannot make definitive conclusions about the underlying mechanism by which cocaine self-administration may lead to increases in membrane excitability and resistance.

It is worth noting that all LHb neurons demonstrate clear rebound spiking following even brief and relatively weak hyperpolarizing current injections, as has been previously reported (Wilcox et al., 1988, Gutnick and Yarom, 1989, Chang and Kim, 2004, Weiss and Veh, 2011).

This rebound spiking appeared to be dependent on the magnitude and length of the hyperpolarization stimulus and could last up to 30 seconds following a single hyperpolarizing period in some cases. This firing mechanism is especially interesting at the level of the circuit because it potentially allows both incoming excitatory and inhibitory signals to elicit subsequent excitatory output from LHb neurons. This scenario has interesting implications regarding how plasticity develops within LHb pathways and how it may affect functional output and behavior. This question has apparently been under-explored to this point but seems to merit further investigation to determine the role of LHb rebound spiking in vivo. Interestingly, the rebound spiking patterns did not necessarily match the spiking patterns during depolarization within the same cell. Some cells which showed RS spike patterns during depolarizing current injections then showed BF rebound spiking patterns, despite resting at a constant membrane potential.

LHb neurons are known to fire spontaneously in slices. However, observed spontaneous tonic firing in LHb neurons was highly variable. ~30% of patched cells would demonstrate no spontaneous tonic firing, ~40% of cells would initially show spontaneous tonic activity but would progressively lose that activity within 20 minutes, and ~30% of cells showed consistent spontaneous tonic firing activity lasting for 30+ minutes. When present, spontaneous tonic activity generally occurred at 0.1-10 Hz for standing spikes or at 0.1-2 Hz for tonic bursts. However, even in cells which showed consistent spontaneous tonic firing, the firing rate for an individual cell often fluctuated between higher and lower frequency over time. For these reasons, attempts to measure a reliable and consistent rheo base or an average tonic firing rate were deemed to be too volatile and inconsistent within the population of sampled LHb cells to make any reliable interpretations or conclusions. Other studies have noted similar levels of heterogeneity or variability regarding spontaneous firing in LHb cells (Chang and Kim, 2004,

Weiss and Veh, 2011). No obvious differences in spontaneous tonic firing patterns or rates between LHb cells of cocaine and saline trained animals were apparent.

The VTA-to-LHb pathway is especially interesting because the projecting neurons from the VTA have been shown to possess the TH marker for dopamine production while also possessing markers for GABA and glutamate release (Stuber et al., 2010, Stamatakis et al., 2013). There is evidence that these TH-positive VTA-to-LHb fibers do not actually release dopamine upon stimulation, but rather release GABA or glutamate in mice (Stamatakis et al., 2013). On the other hand, cocaine has been shown to directly affect dopamine signaling within the LHb via D2 and D4 receptors in rats (Good et al., 2013). The full picture of VTA-to-LHb signaling has yet to be revealed, but cocaine-induced changes to intrinsic membrane properties of LHb neurons would likely affect signaling sent along this peculiar pathway. These intrinsic membrane adaptations would broadly affect signal transduction at LHb neurons across all input pathways.

The results of the current study may also have implications beyond cocaine and addictive drug-use because LHb activity also appears to modulate other reward behaviors such as sucrose intake (Friedman et al., 2011), presumably via similar pathways involving the RMTg and VTA. As the present study did not test rewarding stimuli other than cocaine, it is possible that LHb neurons have increased membrane excitability following other rewarding stimuli. In future experiments, it would be interesting to see how LHb excitability is affected by other drugs or sucrose compared to cocaine and saline.

Taken together, the present study characterizes LHb neurons in the parvocellular and central areas of the medial LHb at various time points after saline or cocaine self-administration and determines that LHb neurons have higher membrane excitability 1-7 days after cocaine self-

administration. This effect returned to baseline by 45 days of withdrawal from cocaine. This change in cell excitability was correlated with changes in membrane resistance. As the LHb largely sends glutamatergic output to regions including the RMTg, which then sends GABAergic projections to the VTA, this result has potentially important implications in the sensitization of negative affect and opponent processes following addictive drug exposure (Solomon and Corbit, 1974, Koob and Le Moal, 2008), especially if this window of LHb signal amplification is sufficient to trigger additional long-lasting circuitry adaptations. Preventing or reversing these LHb neuron adaptations after cocaine exposure may assist in reducing chronic negative affect and reduce relapse.

Table 1. Characteristics of LHb Regular Spiking (RS) Neurons 1 to 2, 5 to 7, and 45 Days after 5-Day Cocaine or Saline Self-Administration

		Current Steps (pA): Voltage difference (mV)					Current Steps (pA): Average Spike Number											mV from Threshold	
RS LHb Cells		-50	-40	-30	-20	-10	+10	+20	+30	+40	+50	+60	+70	+80	+90	Mem. Resist. (MΩ)	Threshold (mv)	fAHP (mV)	mAHP (mV)
Saline 1-2d	n=19	-27.47	-22.03	-18.00	-13.22	-7.32	3.00	4.40	6.72	8.14	9.50	10.26	11.47	12.74	13.58	618.60	-35.17	-23.50	-25.18
SEM		3.56	2.36	2.00	1.72	0.93	0.81	0.97	0.96	1.04	1.02	1.06	1.06	1.26	1.23	73.49	1.19	1.79	1.93
Cocaine 1-2d	n=19	-41.55	-33.96	-26.47	-19.07	-10.37	3.29	5.33	7.15	9.24	10.78	12.60	**13.88**	**15.56**	**16.32**	**910.53**	-34.04	-23.42	-27.13
SEM		5.25	3.73	3.10	2.55	1.58	0.50	0.50	0.58	0.69	0.76	0.92	0.94	1.07	1.08	115.91	1.07	1.70	1.83
Saline 5-7d	n=18	-34.89	-27.88	-20.32	-14.50	-7.79	2.75	4.89	6.04	7.46	8.62	9.65	10.95	12.22	13.27	632.37	-33.94	-24.43	-25.02
SEM		3.37	2.77	2.27	1.70	1.03	0.52	0.56	0.64	0.72	0.69	0.78	0.80	0.89	0.99	51.03	0.75	0.86	0.97
Cocaine 5-7d	n=19	-35.44	-30.45	-24.07	-17.34	-10.24	2.89	5.47	7.89	9.69	11.32	12.26	**14.02**	**15.64**	**17.01**	**863.36** *	-36.00	-24.38	-24.75
SEM		2.88	2.59	1.87	1.36	1.02	0.36	0.45	0.54	0.51	0.59	0.68	0.81	0.87	1.11	72.13	0.99	1.21	0.93
Saline 45d	n=10	-35.43	-29.38	-20.89	-14.96	-8.74	2.50	4.33	6.05	7.55	8.60	10.15	11.30	12.57	13.47	732.42	-37.52	-23.84	-25.24
SEM		1.25	0.94	1.44	0.93	0.44	0.46	0.55	0.54	0.59	0.56	0.68	0.93	0.73	0.73	34.93	1.29	1.56	1.45
Cocaine 45d	n=15	-45.55	-36.44	-29.16	-20.16	-11.45	2.72	4.57	5.97	7.29	8.99	10.43	12.12	13.49	14.92	814.13	-36.46	-24.61	-25.57
SEM		2.87	2.95	2.43	2.13	1.40	0.29	0.48	0.62	0.67	0.83	0.96	1.07	1.24	1.31	52.57	1.20	1.29	1.59

Threshold denotes the voltage point when a cell's voltage begins the final curve upwards to form the action potential peak at >25 mV/ms; bolded entry, $P < .05$ vs saline controls; *, $P < .01$ vs saline controls.

4.0 COCAINE ALTERS SIGNALING WITHIN THE PARAVENTRICULAR NUCLEUS OF THE THALAMUS-TO-NUCLEUS ACCUMBENS PATHWAY

4.1 OVERVIEW AND INTRODUCTION

Drug Addiction is characterized by maladaptive changes in signaling between brain regions which regulate rewards and motivated behaviors. The paraventricular thalamic nucleus (PVT) is a brain region which sends direct projections to the nucleus accumbens (NAc) and contributes to addictive behaviors. We sought to characterize the molecular and cellular changes within the PVT-to-NAc pathway in response to cocaine self-administration. We used virally mediated channelrhodopsin injections in the PVT of rats to isolate fibers from the PVT while recording from neurons in the NAc shell. We found that cocaine self-administration increases silent synapses within the PVT-to-NAc pathway. Additionally, calcium-permeable AMPARs are present at PVT-to-NAc synapses under normal conditions, but are not recruited to maturing silent synapses. Cocaine self-administration also leads to a greater probability of presynaptic vesicle release, which persists though long-term withdrawal. After long-term withdrawal, the number of presynaptic release sites within the PVT-to-NAc pathway is reduced, but the quantal size of presynaptic vesicles is increased, suggesting that the fewer remaining synaptic connections are stronger. We then examined the mechanism of origin of the observed cocaine-generated silent synapses within this pathway and found that silent synapses are most likely

generated from the internalization of AMPARs at existing synapses. These results are the first to characterize cellular and molecular signaling along the PVT-to-NAc pathway in the context of cocaine exposure.

Drug addiction is a disorder characterized by chronic relapse to drug-seeking behavior. Drug-induced changes to glutamatergic neural circuitry related to motivated behaviors and reward is critically implicated in mediating relapse and addictive behaviors (Kalivas, 2004, Wolf and Ferrario, 2010, Pickens et al., 2011). Specifically, medium spiny neurons (MSNs) within the nucleus accumbens (NAc) shell mediate emotional and motivational arousal leading to behavioral output (Kelley and Berridge, 2002). Glutamatergic input to NAc MSNs is targeted by drugs of abuse, such as cocaine, to produce adaptive changes (Wolf, 2010). These drug-induced signaling adaptations may substantially reshape the functional output of NAc MSNs, leading to the prioritization of addiction-related behavioral output.

The generation of silent synapses is another method of cocaine-initiated signaling adaptation (Huang et al., 2009). Silent synapses are thought to be immature synaptic connections which contain NMDA receptors (*N*-methyl-D-aspartate receptor, NMDARs), but lack a normal compliment of AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor**,** AMPARs) (Groc et al., 2006, Kerchner and Nicoll, 2008, Hanse et al., 2013). Maturation of these silent synapses involves the recruitment of AMPARs, and in some cases calcium-permeable AMPARS (CP-AMPARs) (Wolf and Tseng, 2012, Lee et al., 2013, Ma et al., 2014), to the synaptic membrane and may alter the flow of neural information within the affected circuits (Wolf, 2010, Wolf and Ferrario, 2010). Our lab has also recently demonstrated that the maturation of cocaine-generated silent synapses in pathways to the NAc is critical for regulating the incubation of cocaine craving and re-exposure cocaine-seeking (Lee et al., 2013, Ma et al.,

2014). Examination of cocaine-generated silent synapses and their maturation in the NAc may reveal neural and signaling mechanisms that contribute to addictive behaviors (Lee and Dong, 2011).

One potentially important, yet less-examined, source of glutamatergic input to the NAc is the paraventricular nucleus of the thalamus (PVT). The PVT sends direct excitatory projections to the NAc (Van der Werf et al., 2002, Smith et al., 2004) and to the prefrontal cortex (PFC), positioning it well to regulate cortico-striatal signaling involved in motivation and reward (Berendse and Groenewegen, 1990, O'Donnell et al., 1997, Otake and Nakamura, 1998, Cardinal et al., 2002, Parsons et al., 2007, Li and Kirouac, 2008, Vertes and Hoover, 2008). Perhaps unsurprisingly then, the PVT has been shown to mediate cocaine-related behaviors: inactivation of the PVT prevents cocaine-prime induced reinstatement (James et al., 2010), sensitization to cocaine (Young and Deutch, 1998), and expression of cocaine-induced conditioned-place preference (Browning et al., 2014). Additionally, PVT neurons are activated by cocaine-paired contextual cues (Brown et al., 1992, Franklin and Druhan, 2000), and inactivation of hypocretin receptor 1 in the PVT prevents cocaine seeking but not natural reward seeking (Martin-Fardon and Weiss, 2014).

The current study sought to examine pre and postsynaptic signaling within the PVT-to-NAc pathway after cocaine or saline self-administration after short-term (1-2 days) and long-term (43-47 days) withdrawal. We found that the PVT-to-NAc pathway may contain a relatively high baseline level of silent synapses, but that cocaine self-administration generates additional silent synapses. AMPA/NMDA ratio examinations corroborated this finding. About 20% of AMPARs in the PVT-to-NAc pathway of control animals appear to be CP-AMPARs, but long term withdrawal from cocaine and the maturation of cocaine-generated silent synapses does not

appear to affect this percentage. Cocaine also significantly affects presynaptic signaling at PVT-to-NAc synapses. Cocaine increases the probability of vesicle release from presynaptic terminals, and long-term withdrawal from cocaine reduces the total number of presynaptic release sites, while also strengthening remaining synapses by increasing the quantal size of released vesicles. We then determined that cocaine-generated silent synapses within the PVT-to-NAc pathway are derived from the internalization of existing AMPARs.

Thus, cocaine self-administration may remodel the PVT-to-NAc pathway, disrupting normal signaling and causing the PVT to contribute to addictive behaviors. This is first study to examine how cocaine affects the cellular and molecular signaling properties of the PVT-to-NAc pathway.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male Sprague-Daley Rats were ordered from Charles Rivers Labs weighing 90-110 grams upon arrival. Rats were housed in pairs with 12 h/12 h light/dark cycles and free access to food and water. Animals were allowed to habituate to their cages for at least 5 days before undergoing any procedures. Following surgical procedures, animals were single-housed. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University and were performed in accordance with the guidelines of the National Institutes of Health.

4.2.2 Surgeries

After habituating to their cages, p33-40 rats (weighing between 125 and 145 grams) underwent self-administration catheter surgery and stereotaxic virus delivery surgery in a single surgical session. Rats were anesthetized with a ketamine/xylazine mixture (50–100/5-10 mg/kg, i.p.). A silicone catheter (0.51 inner/0.940 mm outer diameter, HelixMark) was then inserted into the jugular vein and run under the skin to a small incision made between the scapulae where it exited the body and connected to a harness with quick connect luer (SAI infusion technologies) worn by the rat. Throughout the recovery and training period, catheters were flushed daily through the harness with sterile saline solution containing gentamicin (5 mg/mL) and heparin (10 μs/mL).

Immediately following catheter surgery, rats undergoing viral injections were placed in a stereotaxic apparatus (Stoelting). A 28-gauge injection needle was used to inject 1.5 μL (0.3 μL/min) of the AAV-ChR2YFP solution via a Hamilton syringe and Thermo Orion M365 pump (Thermo Scientific) into the paraventricular nucleus (AP -3.05, ML ±0.00, DV −5.55). In later experiments, a 28-gauge injection needle was used to also bilaterally inject 1 μL (0.2 μL/min) of AAV-GluA2eGFP-3A or 3Y solution into the nucleus accumbens shell (AP +1.55, ML ±0.80, DV −7.05). Injection needles were left in place for 5 min after injection.

4.2.3 Virus Preparation

Channelrhodopsin-2 (ChR2, H134R variant) fused to Venus (Addgene plasmid 20071) was under the control of a CAG promoter in an adeno-associated viral vectors (AAV) with AAV2 ITRs: AAV-ChR2Y (Mattis et al., 2012). AAV1/2 serotype AAV vectors were generated adenovirus free essentially as described in (Klugmann et al., 2005, Pilpel et al., 2009). Briefly,

human embryonic kidney 293T cells were transfected with the AAV-ChR2 plasmid, AAV1 (pH21) and AAV2 (pRV1) helper plasmids, and the adenovirus helper plasmid (pFΔ6) by standard polyethylenimine transfection. Cells were harvested 48–72 h after transfection and purified by discontinuous iodixanol gradient centrifugation (20). After desalting by Amicon 100k concentrators (Millipore) with PBS-MK according to manufacturers' procedures, AAV vectors were stored at -80 °C until further use.

Generation of the 3A and 3Y GluA2 C-terminal tail plasmids (AAV-GluA2eGFP-3A or 3Y) was done via a similar process. The relevant GluA2 C-terminal tail sequences of these plasmids are as follows: 3A, TAKEGANVAGIESVKI; 3Y, TYKEGYNVYGIESVKI. Both viruses are similar, but have a difference of 1 amino acid in 3 locations (Y-A swap). The 3A variant is an inactive mutant control, while the 3Y variant is active. Both were fused to a GFP label, which was also visible under 512 nm light.

4.2.4 Behavioral Training

Following surgery, the rats were placed back in cages to be single-housed and allowed to recover for 13-18 days before beginning self-administration training. Self-administration operant chambers (Med Associates) contained 2 separate nose-poke holes 6 cm above the grid floor. The harness luer was attached to a swivel with a tether and connected to a syringe loaded into an infusion pump. Nose pokes to the active hole initiated an infusion "reward" of cocaine/saline (0.75mg/kg cocaine or an equivalent volume of saline over 6 s; the volume of each infusion was 90-150 µL and was adjusted based on the body weight of the animal at each training session), turned off the house light for 20 s, and turned on a separate 6 s cue light. A successful infusion was followed by a 14 s lockout period. During this lockout period, active nose pokes continued

to be recorded but failed to initiate any cues or further infusions. At the end of this lockout period, the house light would turn back on, signaling that the active nose poke hole could now initiate another infusion. Nose pokes in the inactive hole were recorded but elicited no effects. All tests were done using an FR1 schedule.

Rats were randomly divided into cocaine and saline treatment groups and were placed in the operant chambers for one over-night training session (~12 hours) 13-18 days after surgery. Rats in cocaine groups that failed to receive greater than 30 cocaine infusions during the overnight session were excluded from further testing (~20% of the group). ~24 hours later, rats began a series of daily 2-hour training sessions over 5 consecutive days. Rats in a cocaine group which did not show the ability to distinguish between active and inactive nose pokes or did not receive at least 12 cocaine infusions per 2 hour session were excluded from further study (~10% of the group). At the conclusion of the training, rats from all groups were placed back into their home cages for 24-48 hours or 43-47 days for long-term withdrawal groups before being taken for brain slice preparation.

4.2.5 Brain Slice Preparation

Rats were quickly anesthetized with ~3-4 mL of 99.9% isofluorane in a closed 20x16x16 cm chamber and were decapitated so that the brain could be extracted. Coronal brain slices (260 µm thick) containing the PVT or the NAc were prepared using a VT1200S microtome (Leica). Slices were cut in the presence of 4°C cutting solution containing (in mM): 135 *N*-methyl-D glucamine, 1 KCl, 1.2 KH_2PO_4, 0.5 $CaCl_2$, 1.5 $MgCl_2$, 20 choline-HCO_3, and 11 glucose, saturated with 95% O_2/5% CO_2, with the pH adjusted to 7.4 using HCl, at 300-310 mOsm. After being cut, slices were placed in an incubation chamber in artificial cerebrospinal fluid (aCSF) containing

the following (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, at 290-294 mOsm, saturated with 95% O2/5% CO_2 to maintain a pH of 7.4 at 37°C for 30 min. Slices were then allowed to recover for at least 30 min at room temperature before being used for experimentation.

4.2.6 Electrophysiological Recordings

Whole-cell voltage-clamp recordings were made in the NAc shell. During recordings, slices were superfused with aCSF that was heated to 31–33°C by passing the solution through a feedback controlled in-line heater (Warner Instruments) before entering the recording chamber. Recordings were made under visual guidance (40x, differential interference contrast optics) with micropipettes (2.5–5 MΩ) filled with a cesium-based internal solution containing (in mM): $CsCH_3O_3S$ (140), tetraethylammonium chloride (TEA-Cl) (5), EGTA (0.4), Hepes (20), Mg-ATP (2.5), Na-GTP (0.25) and QX-314 (1), pH 7.3, 294 mOsm.

To evoke PVT-to-NAc synaptic transmission, axons expressing ChR2 were stimulated by 473 nm DPSS laser (IkeCool) coupled to a 62.5 μm optic fiber, generated using Clampex software (Molecular Devices). Collimated laser light was coupled to a fluorescent port of the Olympus BX51WI microscope, allowing the blue laser light to illuminate the slice through the objective, focused on the cell. An optical stimulation of 1.0-0.05 ms duration was used to stimulate at 0.1 Hz for paired-pulse or AMPA-NMDA ratio measurements, 0.04 Hz for trains of 5 pulses (50 ms inter-pulse interval), and 0.17 Hz for minimal stimulation measurements. To record excitatory postsynaptic currents (EPSCs), picrotoxin (100 μM) was included in the external aCSF to block GABAA. Series resistance was 8 – 20 MΩ, uncompensated, and monitored continuously during recording. Recordings with a change in series resistance greater

than 20% for the duration of data collection were not accepted for analysis. Current traces were recorded with a MultiClamp 700B amplifier (Molecular Devices), filtered at 3 kHz, amplified 5 times, and then digitized at 20 kHz. Cells were allowed ~5 minutes to stabilize after achieving the whole-cell patch configuration before beginning data collection.

Stimulation intensity for minimal stimulation protocols was set by adjusting stimulator output to elicit EPSC responses in approximately 30–70% of trials at −70mV and was then kept constant through changes in holding potential. Stimulation intensity during other trials was adjusted to evoke EPSC responses preferentially at 100-300 pA.

4.2.7 Staining and Imaging

Injections of biotinylated dextran amine (BDA; 10,000 molecular weight, Molecular Probes, dissolved as a 10% solution in 10 mM sodium phosphate buffer) were done into the PVT at 2.4 mm posterior to Bregma, 1.1 mm lateral to the midline, and 5.45 mm ventral to the skull surface by using a 14-degree angle relative to the midline vertical axis (Paxinos and Watson, 1997). The tracer was delivered by iontophoresis through a glass pipette with 50- to 75-μm tip diameter using a positive 5 μA current pulsed 10 seconds on and off for a total of 30 minutes. The pipette was then left in place for an additional 5 minutes. After 5-days of recovery, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Tissue from these rat brains were used for multiple purposes across labs. One of these purposes required the chelation of endogenous zinc. Consequently,, animals receiving BDA injections were subsequently treated for 15 minutes with 1 g/kg, i.p. of a zinc chelator, diethyldithiocarbamic acid, (Sigma) (Veznedaroglu and Milner, 1992). All rats were perfused through the aorta with 10 ml of a heparin-saline solution (1,000 U/ml heparin in 0.9% saline) followed by 50 ml of 3.75% acrolein in 2% paraformaldehyde, and finally 250 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were extracted, post-fixed in 2% paraformaldehyde

for 1 hour, and sectioned at approximately 50 μm by using a Vibratome. Sections were then mounted on glass slides, dehydrated, and cover-slipped. Pictures were taken on an Olympus BM51 microscope and were adjusted for contrast and color balance using Adobe Photoshop.

4.2.8 Drugs and Reagents

Cocaine-HCl was supplied by the Drug Supply Program of the National Institute of Drug Abuse. All other chemicals were purchased from Sigma-Aldrich.

4.2.9 Statistics and Analysis

All results are shown as mean ± SEM. Minimal stimulation assay (Isaac et al., 1995, Liao et al., 1995, Huang et al., 2009) was used to measure the ratio of silent synapses. The percentage of silent synapses within the measured environment can be estimated by the formula:

$$\text{Silent synapse ratio} = 1-\ln(\text{failure rate}_{-70}) / \ln(\text{failure rate}_{+50})$$

The NMDAR-mediated component was recorded at +50 mV. The amplitude was operationally defined as the amplitude of the current 40 ms after the onset of the evoked current, at a time point when AMPAR-mediated currents have subsided.

For variance-mean analysis (multiple probability fluctuation analysis, MPFA), 30–100 AMPAR EPSCs were used from each cell at five release probability conditions achieved by a five-pulse train of presynaptic stimulations with a short interpulse interval (50 ms) (Scheuss and Neher, 2001, Silver, 2003). The peak amplitude of each EPSC was compared to its baseline and

averaged. Variance of EPSC peaks was calculated and plotted against their mean amplitude for each eliciting condition. We assumed the presynaptic releasing sites are independent, and the release probability across all synapses within the examined pathway is the same in each cell. Thus, the amplitudes of EPSCs can be expressed as:

$$I = NPrQ \qquad [EQ\ 1]$$

N is the number of release sites, Pr is the presynaptic release probability, and Q is the quantal size (amplitude of postsynaptic response upon a release of one quantum). For a binomial model, the variance (σ2) of EPSC amplitudes can be expressed as:

$$\sigma^2 = NQ^2Pr(1-Pr) \qquad [EQ\ 2]$$

Based on these 2 above equations, the following equation can be derived:

$$\sigma^2 = IQ-(1/N)^2 \qquad [EQ\ 3]$$

This equation predicts a parabolic relationship between σ^2 and I. As such, the variance-mean relationship was fit with EQ 3 to estimate N, and Q in each examined cell. Pr was then calculated with EQ 1. If one of the theoretical assumptions does not hold (e.g., if multivesicular release exists), the σ^2-I curve would not exhibit such a parabolic relationship. 22 cells among the total of 70 recorded cells undergoing 20 Hz stimulation were not included in the final data analysis because they could not be well-fitted by this relationship ($R^2 < 0.9$), or they had minimal variance in EPSCs. In a few cells (n = 3), only the first four EPSCs were included in the analysis because the fifth EPSC was predominated by failed responses. Student's Ttest was used to

compare averaged data points between groups, and paired Ttest was used to compare before-after data points within subjects.

4.3 RESULTS

4.3.1 Paraventicular Thalamic Nucleus Sends Projections Primarily to the Nucleus Accumbens Shell

The PVT is a long thin structure extending ventrally adjacent along the third ventricle. The direct pathway between the PVT and the NAc has been described previously (Christie et al., 1987, Otake and Nakamura, 1998, Pinto et al., 2003, Vertes and Hoover, 2008), and reports also show a distinction in the projections between the anterior and posterior PVT. The anterior PVT projects more to the NAc core while the posterior PVT projects more to the NAc shell (Vertes and Hoover, 2008). To ensure that our injection/infection location within the PVT adequately innervated the NAc, we injected BDA into the more posterior PVT and then looked for fibers in the NAc (**Fig. 11a, b**). We found good innervation within the NAc shell, which was also functionally confirmed in subsequent experiments involving pathway stimulation.

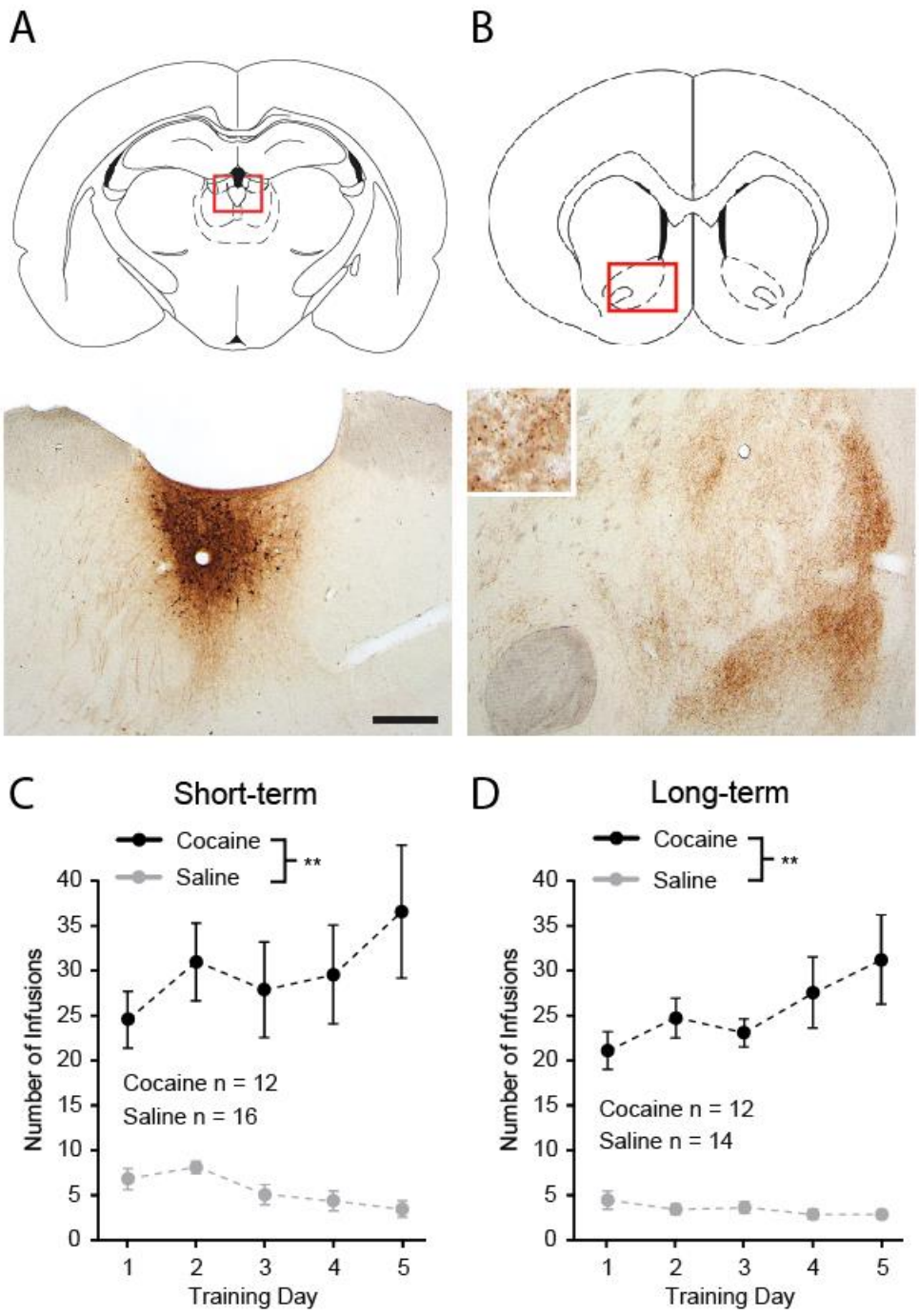

Figure 11. BDA tracing of PVT-to-NAc fibers.
(**a**) Location of BDA injection within the PVT. (**b**) Stained PVT fibers visible in the NAc shell from the injection shown in (a). Note the punctate nature of the labeling in the boxed region shown at higher magnification in the insert. Scale bar = 250 μm for panels (a) and (b), and 25 μm for the inset in (b). (**c, d**) Number of infusions of saline and cocaine for animals in short-term (c) and long-term (d) withdrawal groups during the 5 daily 2-hour self-administration sessions.

4.3.2 Cocaine Self-administration Generates Silent Synapses within the PVT-to-NAc Pathway

Animals that had received viral injections for the expression of ChR2 in the PVT then underwent either cocaine or saline self-administration training (**Fig. 12c, d**). NAc slices were then taken after short-term (1-2 day) withdrawal after 5 days of training. NAc shell MSNs were patched and underwent the minimal stimulation assay (Isaac et al., 1995, Liao et al., 1995, Huang et al., 2009), which takes advantage of the fact that NMDAR-only synapses are inactive at polarized resting potentials due to magnesium block but are active at depolarized potentials because the magnesium block is released. To detect silent synapses only within the PVT-to-NAc pathway, we minimally stimulated PVT fibers with 488 nm light. It was observed that the cells from cocaine-trained animals exhibited significantly more silent synapses than saline-trained animals (**Fig. 12a**). This increase in silent synapses did not persist through long-term (42-47 days) withdrawal, instead returning to baseline levels (**Fig. 12b**). This suggests that cocaine does generate silent synapses within the PVT-to-NAc pathway, similar to other pathways which have been specifically measured (Lee et al., 2013, Ma et al., 2014). Interestingly, the baseline level of silent synapses within the PVT-to-NAc pathway does appear to be higher (~20%) compared to other pathways such as the prefrontal cortex and amygdala or random sampling including all pathways (~5-10%). Nevertheless, cocaine exposure generates a proportional increase in silent synapses in connections coming from the PVT, despite higher basal levels. Silent synapses are no longer detected after long-term withdrawal from cocaine because many of the previously silent synapses have presumably matured into functional synapses containing AMPARs.

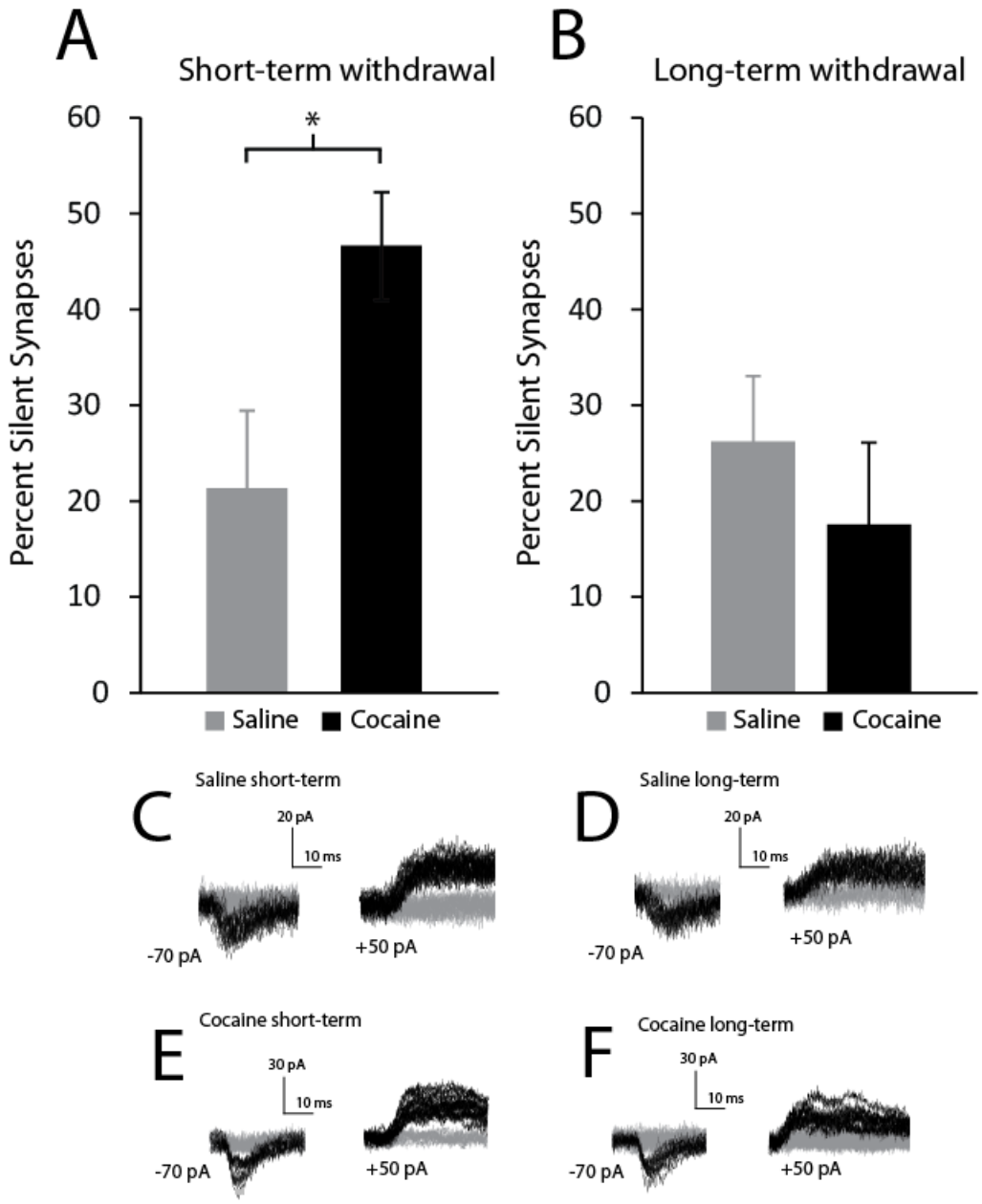

Figure 12. Cocaine self-administration generates silent synapses within the PVT-to-NAc Pathway after short-term withdrawal.

(**a**) Percentage of silent synapses of all synapses within the PVT-to-NAc pathway after short-term withdrawal from 5-day cocaine or saline self-administration training ($p < 0.05$, n sal = 10, n coc = 17). (**b**) Percentage of silent synapses of all synapses within the PVT-to-NAc pathway after long-term withdrawal from 5-day cocaine or saline self-administration training (n sal = 13, n coc = 11). (**c-f**) Overlaid responses (black) and failures (gray) of example traces from the minimal stimulation assay after saline (c) and cocaine (e) self-administration short-term withdrawal and after saline (d) and cocaine (f) self-administration long-term withdrawal.

4.3.3 AMPA/NMDA Ratio in PVT-to-NAc Pathway Is Affected by Cocaine Self-administration

To confirm and corroborate the minimal stimulation data, we also examined the AMPA/NMDA ratio of NAc MSNs. By stimulating PVT-to-NAc shell connections, we found that animals trained to self-administer cocaine showed a significant decrease in the AMPA/NMDA ratio (**Fig. 13a**). This result fits with the increase in silent synapses at the same stage, as all of the detected silent synapses contain NMDARs without AMPAR compliment. And, just as the silent synapse levels return to baseline after long-term withdrawal, the AMPA/NMDA ratios do as well (**Fig. 13b**). This result showing a decrease in AMPA/NMDA ratio can be due either to an increase in synaptic NMDAR content or a decrease in synaptic AMPAR content. This result helps to confirm that silent synapses are indeed generated, but it does not help in determining the mechanism of generation. Silent synapses can be generated by either adding NMDARs to new synapses or by removing AMPARs from existing synapses. Both mechanisms would result in a reduction in AMPA/NMDA ratio, such as observed here.

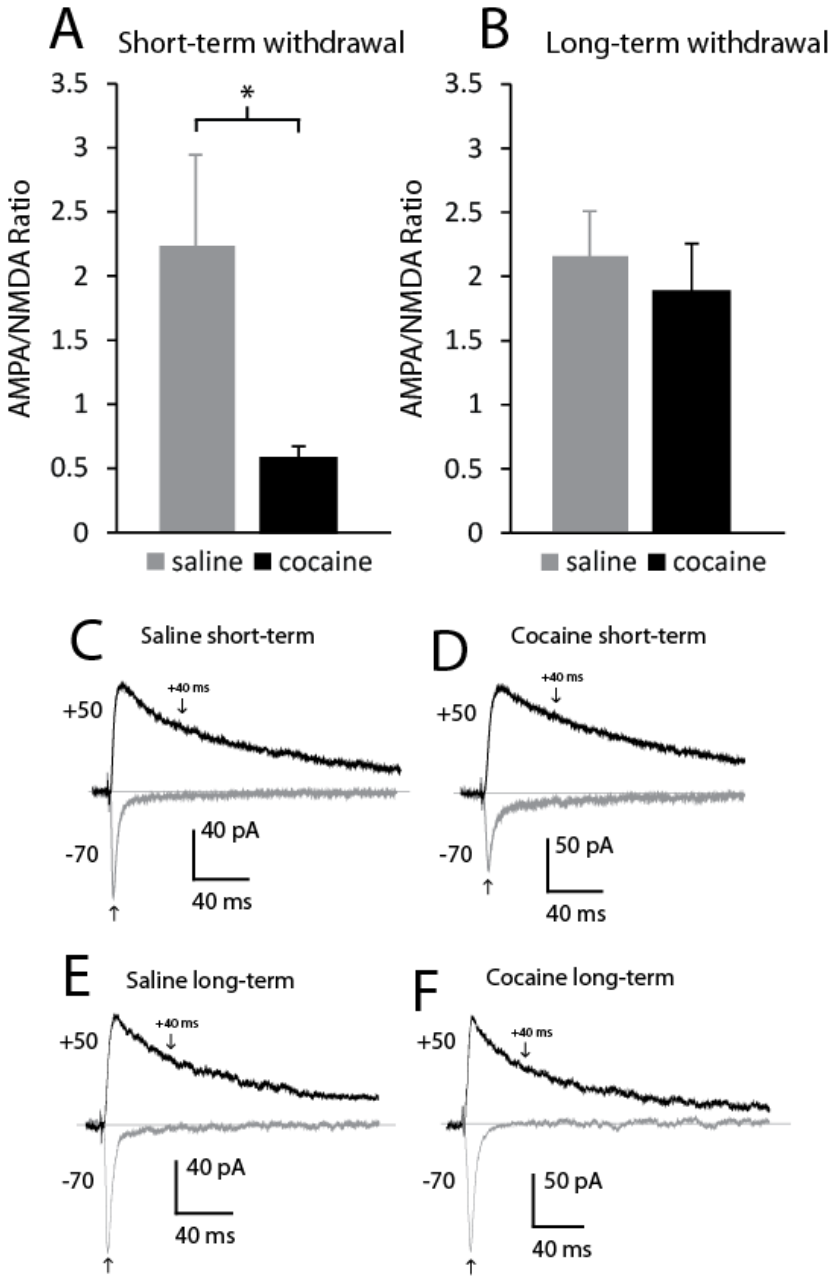

Figure 13. Cocaine self-administration reduces the AMPA/NMDA ratio at PVT-to-NAc synapses after short-term withdrawal.
(**a**) AMPA/NMDA ratio at synapses within the PVT-to-NAc after short-term withdrawal, showing that short-term withdrawal from cocaine decreases the AMPA/NMDA ratio compared to saline controls ($p < 0.05$, n sal = 6, n coc = 7). (**b**) After long-term withdrawal from cocaine, the AMPA/NMDA ratio returns to saline control levels (n sal = 8, n coc = 11). (**c-f**) Averaged example EPSCs at +50 (black) and -70 mV (gray) taken after saline (c) and cocaine (d) self-administration short-term withdrawal and after saline (e) and cocaine (f) self-administration long-term withdrawal.

4.3.4 CP-AMPARs Are Not Inserted at Maturing Cocaine-generated Silent Synapses within the PVT-to-NAc Pathway

After withdrawal from cocaine self-administration, silent synapses in the NAc may mature by recruiting calcium-permeable (CP)-AMPARs to the membrane (Conrad et al., 2008, Wolf and Tseng, 2012, Lee et al., 2013). CP-AMPARs are not often detected at NAc MSNs under normal conditions. However, CP-AMPARs appear in the NAc after addictive drug exposure and mediate aspects of addictive behavior, including the incubation of cocaine craving (Conrad et al., 2008, Wolf and Tseng, 2012, Lee et al., 2013). Our lab has recently published an example of differences in the recruitment of CP-AMPARs in different pathways (Ma et al., 2014), and we were interested in whether cocaine-generated silent synapses within the PVT-to-NAc pathway would also demonstrate the recruitment of CP-AMPARs after 45 days of withdrawal.

Rats trained to self-administer either cocaine or saline were tested after short-term withdrawal or long-terms withdrawal. MSNs were patched, and a stable synaptic response was established at PVT-to-NAc synapses. A CP-AMPAR-selective antagonist was then washed in via local perfusion while the amplitude of the responses continued to be measured. Somewhat surprisingly, the NASPM application blocked ~20% of the synaptic response in both saline and cocaine treated rats after short-term withdrawal (**Fig. 14a**). After long-term withdrawal, NASPM application continued to block responses by ~20% in both saline and cocaine treated animals (**Fig. 14b**). These results indicate that roughly 20% of synaptic AMPARs within the PVT-to-NAc pathway are CP-AMPARs under normal (cocaine naïve) conditions—a higher percentage than detected in other pathways within the NAc—and that cocaine treatment and the maturation of cocaine-generated silent synapses does not lead to any increase in CP-AMPAR expression within this pathway.

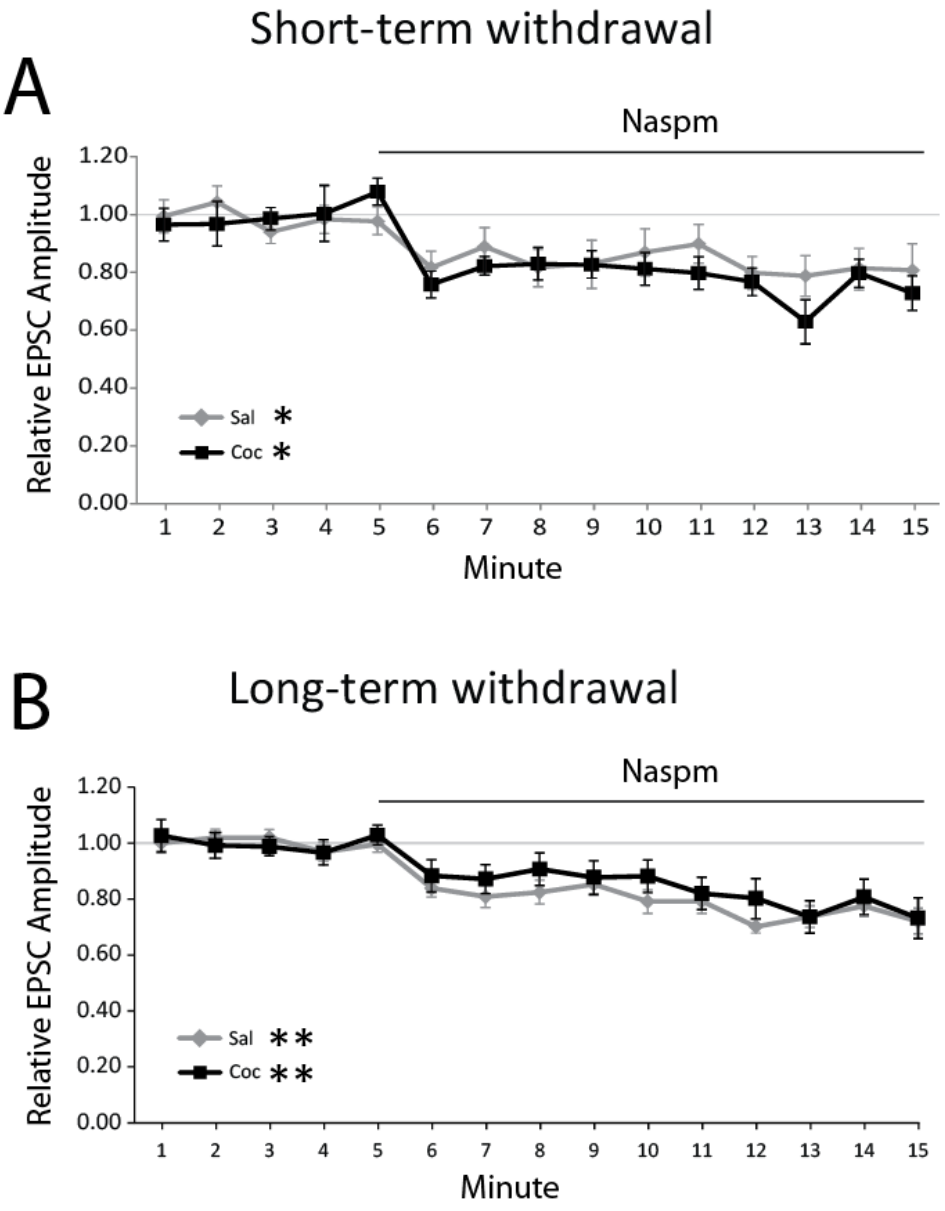

Figure 14. CP-AMPARs are present within the PVT-to-NAc pathway, and are unaffected by cocaine self-administration.

(**a**) Local perfusion of Naspm leads to ~20% reduction in average EPSC amplitude at PVT-to-NAc synapses after short-term withdrawal from either saline or cocaine self-administration. Comparisons were made between average amplitudes at minutes 1-5 vs. 5-minute sections with Naspm (sal, $p < 0.05$, $n = 12$; coc, $p < 0.05$, $n = 5$). (**b**) Local perfusion of Naspm also leads to ~20% reduction in average EPSC amplitude at PVT-to-NAc synapses after long-term withdrawal from saline or cocaine self-administration (sal, $p < 0.01$, $n = 20$; coc, $p < 0.01$, $n = 12$). *, $p < 0.05$; ** $p < 0.01$.

4.3.5 Cocaine Self-administration Alters Presynaptic Signaling Properties within the PVT-to-NAc Pathway

Just as cocaine may alter postsynaptic characteristics of reward-related pathways, presynaptic characteristics can be modified as well. Previous work has indicated that different reward-related pathways may undergo different presynaptic changes in response to cocaine exposure (Ishikawa et al., 2013, Suska et al., 2013). We sought to examine how cocaine self-administration may change presynaptic signaling properties within the PVT-to-NAc pathway.

As before, rats were split into cocaine and saline treatment groups and examined after either short-term or long-term withdrawal, and blue light was used to stimulate PVT fibers in the NAc shell. To detect presynaptic properties, we employed multiple-probability fluctuation analysis (MPFA, see Methods section 4.3.9). This analysis method allowed us to estimate a range of presynaptic properties under certain conditions. We found that the paired-pulse ratio was changed after cocaine treatment (**Fig. 15a**), indicating that cocaine initiates presynaptic signaling changes within the PVT-to-NAc pathway. The paired-pulse ratio remained similarly altered even after long-term withdrawal (**Fig. 15b**). Analysis based on MPFA revealed that the probability of release was significantly increased after short- and long-term withdrawal in cocaine-trained animals (**Fig. 15c**, **d)**. These results match with the paired-pulse ratio. Further analysis showed that the quantal size of presynaptically released vesicles within the PVT-to-NAc pathway was significantly increased after long-term withdrawal from cocaine, but not short term withdrawal (**Fig. 15e, f**). Thus, the signaling strength of matured silent-synapses may be furthered strengthened within this pathway throughout cocaine withdrawal by packaging greater amounts of neurotransmitter into vesicles. Interestingly, however, the number of presynaptic release points appears to be decreased after long-term withdrawal from cocaine self-

administration, but not short-term withdrawal (**Fig 15g, h**). This is an intriguing result as it suggests that the number of synaptic contacts with this pathway is reduced after extended withdrawal from cocaine. Taken together, it appears synaptic contacts are reduced during withdrawal, but that the remaining synapses have increased signaling strength. These results may provide some support for the idea that cocaine-generated silent synapses within the PVT-to-NAc pathway form from existing synapses via the internalization of AMPARs. However, it could still be possible that silent synapses are formed de novo and mature into strong connections while existing connections, likely related to other memories, are culled away. We sought to answer this question on the origin of cocaine-generated silent synapses in our next set of experiments.

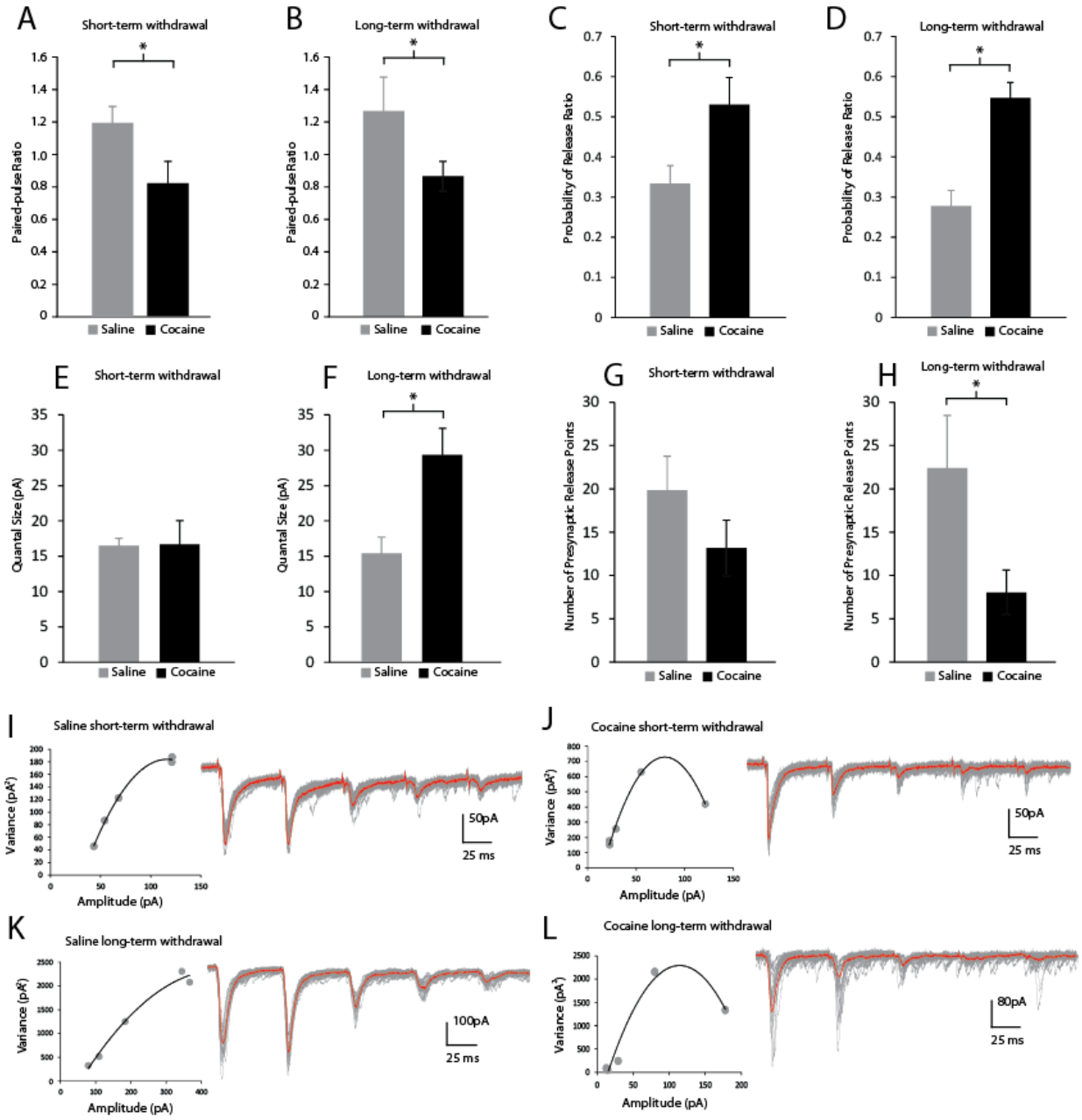

Figure 15. Cocaine self-administration alters presynaptic release properties of PVT-to-NAc synapses.

(**a**) Cocaine self-administration decreases the paired-pulse ratio of PVT-to-NAc EPSCs after short-term withdrawal ($p < 0.05$, n sal = 14, n coc = 8). (**b**) The paired-pulse ratio of PVT-to-NAc EPSCs remains decreased after long-term withdrawal from cocaine self-administration ($p < 0.05$, n sal = 9, n coc = 17). (**c**) The probability of PVT-to-NAc presynaptic vesicle release is increased after short-term withdrawal from cocaine self-administration ($p < 0.05$, n sal = 14, n coc = 8), (**d**) and it remains increased after long-term withdrawal ($p < 0.05$, n sal = 9, n coc = 17). (**e**) The PVT-to-NAc quantal size of is unchanged after short-term withdrawal from cocaine self-administration (n sal = 14, n coc = 8), (**f**) but is increased after long-term withdrawal ($p < 0.05$, n sal = 9, n coc = 17). (**g**) The number of PVT-to-NAc presynaptic release points is

unchanged after short-term withdrawal from cocaine self-administration (n sal = 14, n coc = 8), (**h**) but is significantly reduced after long-term withdrawal ($p < 0.05$, n sal = 9, n coc = 17). (**i-l**) Example data from individual cells showing the variance-amplitude curves derived from 5 stimulations at 20 Hz after short-term withdrawal from (i) saline and (j) cocaine and long-term withdrawal from (k) saline and (l) cocaine.

4.3.6 Cocaine Does Not Generate Silent Synapses if Existing AMPARs Cannot Be Internalized

To determine whether cocaine-generated silent synapses are formed de novo as new synaptic contacts or whether they arise from existing synapses after having internalized their AMPA receptors, we employed an AAV plasmid which expresses a small string of amino acids identical to the C-terminal end of GluA2 subunits found in AMPARs (active '3Y' variant). Thus, cells infected by this virus are flooded by the expression of this small sequence of amino acids, meaning that normal cellular signals interacting with the PDZ domain at the C-terminus of GluA2 subunits should be blocked and sequestered by the over-whelming availability of these small amino acid sequences. These blocked GluA2 PDZ domain interactions include interactions with proteins like GRIP1 and PICK1, which regulate the internalization of AMPARs (Dong et al., 1997, Kim et al., 2001). A second virus, similar to the first, but having different amino acids in 3 locations (Y-A swap), was used as a nonfunctional mutant control (inactive '3A' variant).

Animals were prepared just as in previous experiments, except that an additional set of virus injections was made at the same time as the channelrhodopsin virus injection. Thus, animals additionally received bilateral injection of either the GluA2 C-terminus 3A or 3Y virus to the NAc shell. The timelines for the experiments remained unchanged. Only infected cells within the NAc shell of injected animals were patched and recorded for data. Animals were

trained for cocaine or saline and were examined after short-term withdrawal, when cocaine-generated silent synapses were previously observed within the PVT-to-NAc pathway (**Fig. 12a**).

Animals infected with the 3A virus showed very similar levels of silent synapses as previously observed (**Fig. 16**). However, cells infected with the 3Y virus, showed significantly lower silent synapse levels compared to controls (**Fig. 16**). This result indicates that cocaine-generation of silent synapses requires the internalization of AMPARs, and strongly suggests that silent synapses are formed from existing synapses within the PVT-to-NAc pathway.

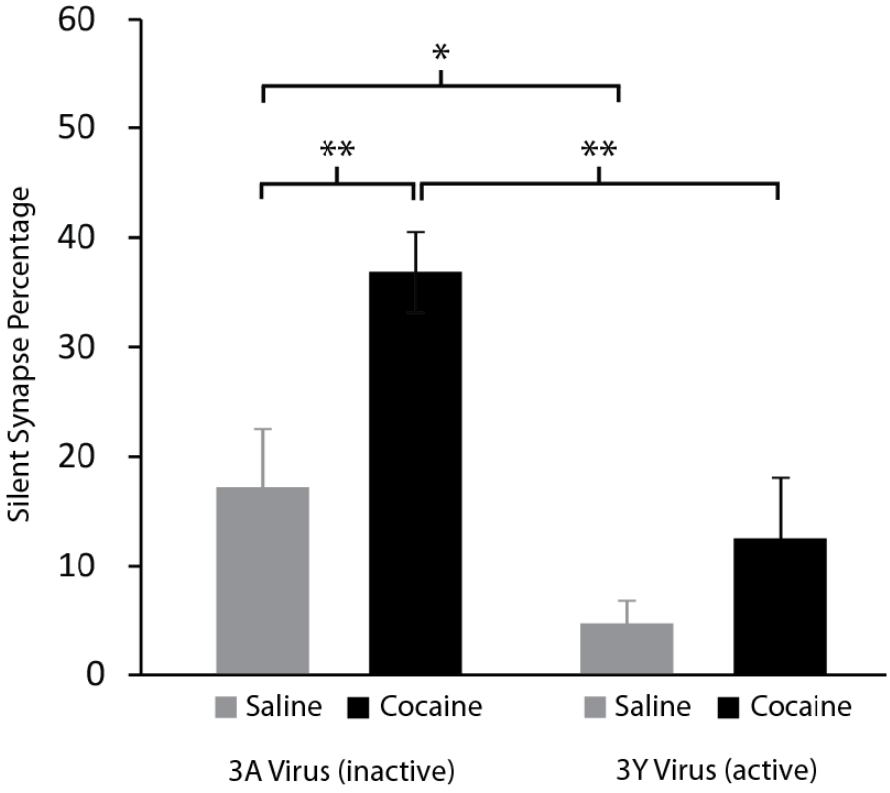

Figure 16. Blocking internalization of AMPARs at PVT-to-NAc synapses also blocks cocaine-generation of silent synapses.
NAc shell MSNs infected with the inactive 3A virus showed cocaine-generation of silent synapses (3A, n sal = 7, n coc = 11), while NAc shell MSNs infected with the 3Y virus (3Y, n sal = 7, n coc = 11) to block and sequester AMPAR internalization signals showed significantly less cocaine-generation of silent synapses as well as lower baseline levels of silent synapses. **, $p < 0.01$; *, $p < 0.05$.

4.4 DISCUSSION

By selectively activating PVT synapses to NAc shell MSNs, we demonstrate that cocaine remodels signaling both pre and postsynaptically within the PVT-to-NAc pathway. This cocaine-based remodeling likely alters the functional output of NAc neurons based on PVT input, potentially contributing to addictive behaviors.

4.4.1 Cocaine Generated Silent Synapses and CP-AMPARs in the PVT-to-NAc Pathway

As the basic unit of communication between neurons, synaptic contacts control signaling patterns and determine circuit functions. Glutamatergic input to NAc MSNs is the major force driving MSN functional output (Wolf, 2010). The present study found high baseline levels (~20%) of silent synapses within the PVT-to-NAc pathway (Fig. 2). For comparison, global sampling within the NAc shell or examination of other pathways shows ~5 to 12% silent synapses (Huang et al., 2009, Koya et al., 2012, Lee et al., 2013, Ma et al., 2014). As silent synapses are thought to be immature synapses which may be substrates for further plasticity (Isaac et al., 1995, Kerchner and Nicoll, 2008, Lee and Dong, 2011), this may indicate that the PVT-to-NAc pathway is able to more readily undergoes plastic changes relative to other pathways. With a greater number of silent synapses present at any time, the PVT-to-NAc pathway may be in a state of high metaplasticity, making it especially sensitive to environmental stimuli (Lee and Dong, 2011).

We also found cocaine-generated silent synapses in the PVT-to-NAc pathway after cocaine self-administration. Because relapse is a critical hallmark of addiction, it has been a

focus of addiction research to identify long-term molecular and cellular changes in the NAc which occur during withdrawal (Conrad et al., 2008, Peters et al., 2008, Wolf, 2010). The appearance of CP-AMARs in the NAc is one such neural adaptation which occurs during withdrawal from cocaine and appears to mediate relapse behavior and the incubation of craving (Conrad et al., 2008, Wolf and Tseng, 2012, Lee et al., 2013, Ma et al., 2014). CP-AMPARs are thought to be recruited specifically to cocaine-induced silent synapses as the synapses mature through withdrawal.

CP-AMPARs lack the GluA2 subunits found in other types of AMPARs and allow for the passage of calcium ions into the cell through their channel, providing an additional source of extracellular calcium which can be activated at sub-threshold voltages (Liu and Zukin, 2007). Normally, GluA2-lacking AMPARs are expressed at very low levels in the NAc and contribute minimally to signaling within the NAc (Conrad et al., 2008). Under normal conditions, ~4-6% of all AMPARs in the NAc are CP-AMPARs (GluA2-lacking) (Conrad et al., 2008, Reimers et al., 2011). However, the present study found that ~20% of PVT-to-NAc AMPARs are CP-AMPARs under normal conditions (Fig. 4). Thus, the small existing amount of CP-AMPARs in the NAc may preferentially be located at PVT-to-NAc synapses, putting baseline levels within other pathways at even smaller percentages than the overall average level of ~5%. This functional expression rate was consistent across both saline and cocaine groups for both short-term and long-term withdrawal.

It is currently unclear why higher basal levels of CP-AMPARs may exist specifically within the PVT-to-NAc pathway, but it may somehow be related to the fact that this pathway also possesses high baseline levels of silent synapses. Previous studies have shown that CP-AMPARs are likely recruited to maturing silent synapses after cocaine, but not in all pathways.

The infralimbic and basolateral amygdala pathways to the NAc recruit CP-AMPARs to silent synapses after withdrawal from cocaine, contributing to the incubation of craving and relapse-like behaviors (Lee et al., 2013, Ma et al., 2014). The prelimbic pathway to the NAc does not recruit CP-AMPARs to maturing silent synapses, and instead inhibits craving and relapse behaviors (Ma et al., 2014). The unique cellular properties of the PVT-to-NAc pathway, including existing basal levels of CP-AMPARs which are not increased as silent synapses mature, make direct comparisons to any other described pathways to the NAc difficult. The basal presence of CP-AMPARs may simply be a side effect due to the relatively high turnover rate within this pathway or may simply be a normal adaptive neural feature which aides in regulating neural information sent from the PVT to NAc. Given these unique properties, it therefore remains an important question how exactly the maturation of silent synapses at PVT-to-NAc synapses affects drug-seeking and relapse behaviors throughout withdrawal from cocaine.

4.4.2 Cocaine Alters Presynaptic Properties of PVT-to-NAc Synapses

In additional to changing synaptic connections and postsynaptic receptor composition via generating silent synapses, cocaine self-administration also alters presynaptic signaling properties of PVT-to-NAc synapses. NAc MSNs integrate incoming glutamatergic information from numerous sources to process their functional output. Here, we are focused specifically on PVT signaling to the NAc. Optical stimulation of ChR2 was used to elicit 5 PVT-to-NAc stimulations at 20Hz and the resulting traces were analyzed using multiple-probability fluctuation analysis (MPFA). This analysis examined paired-pulse ratio (PPR), probability of presynaptic glutamate release (Pr), the quantal size of the glutamate release (Q), and the number of active release sites (N) (Silver, 2003). Similar five-pulse trains have been used to stimulate

excitatory synapses at five repetitive and consistent release states (with different Prs) for analysis with MPFA (Scheuss and Neher, 2001). And, the ability of ChR2 (the same H134R variant also used in this study) to create consistent synaptic activity has been previously tested and verified to be suitable for use in similar MPFA tests (Suska et al., 2013).

In saline-trained animals, we found that the PPR was greater than 1, indicating paired-pulse facilitation. However, in cocaine-trained animals, the PPR was generally lower than 1, indicating paired-pulse inhibition. Differences in paired-pulse ratio generally indicate presynaptic differences. And, fittingly, analysis with MPFA revealed that cocaine-trained animals had a higher rate of presynaptic release. This fits the PPR data because a higher presynaptic release rate would make fewer vesicles available during quick subsequent stimulations. The PPR and the Pr rates were also consistent across saline and cocaine groups after both short-term and long-term withdrawal. This consistency helps to confirm the reliability of the analysis via MPFA, and it also indicates that long-term withdrawal from cocaine does not rectify the change in Pr rate at PVT-to-NAc synapses once initiated by cocaine self-administration.

It should also be noted that the Pr is assumed to be uniform across all release sites (Scheuss and Neher, 2001). In addition to the assumption that (1) Pr is uniform across release sites, MPFA also assumes that: (2) release sites operate independently, (3) release is synchronous, and (4) *Q* is uniform at an individual site and across release sites. Thus, if these 4 assumptions are not met, the amplitude and variance data from the 5-pulse train could not be well-fit to a binomial model (Scheuss and Neher, 2001, Silver, 2003, Suska et al., 2013). This was the case with 22 of 70 total cells which underwent 5-pulse stimulation and MPFA. These 22 cells were not included in data analysis because a close-fitting binomial curve could not be fit,

indicating that the above assumptions were not met. However, in a majority of recorded cells (48 of 70 total), an appropriate binomial curve could be well-fit to the 5 data points, and these cells were used for analysis.

MPFA also revealed that short-term withdrawal from cocaine self-administration does not significantly affect the quantal size of release or the number of presynaptic release points within the PVT-to-NAc pathway. However, following long-term withdrawal from cocaine, the Q is significantly increased and the N is significantly decreased. This data suggests that withdrawal from cocaine self-administration may decrease the number of PVT-to-NAc synaptic connections, but that the synaptic strength of the remaining connections may be greater because larger amounts of glutamate are release upon activation. Importantly, the Q values and N values were consistent across saline short- and long-term withdrawal groups, further indicating the reliability of these measurements.

The number of release sites (N) is highly depended on the number of synapses activated during stimulation of each recording. This number may exhibit high variability that is unrelated to cocaine exposure, but is instead related to the specific activated synaptic network of the individual patched neuron. However, by averaging the N value over multiple cells, comparisons between groups can be made based on average values. It is also important to note that the number of release sites corresponds only to the number of active release sites during the 5-pulse train stimulation, which corresponds selectively to PVT-to-NAc release points due to the optical stimulation method in this case. Thus, if fewer PVT neurons express the ChR2 protein or if lower levels of ChR2 protein are expressed in a certain treatment groups, a difference in the measured N values between groups might also be expected. However, no obvious differences in the AAV-ChR2 infection rate or in the ChR2-YFP fluorescence expression of PVT neurons was observed

between groups, so there is assumed to be no inherent bias in the number of eligible PVT-to-NAc fibers between treatment groups.

Another important point to consider concerning the observed difference in the N value after long-term withdrawal from cocaine self-administration is the generation of silent synapses as observed in Fig. 2. It is unlikely that silent synapses directly interfere with MPFA measurements of presynaptic parameters. The 5-pulse stimulation is performed at a holding potential of −70 mV, and only active synapses contribute to EPSC peaks. Silent synapses are thus not sampled during MPFA because NMDARs are only active at depolarized potentials. However, due to the disappearance and presumed maturation of silent synapses during withdrawal, it is expected that the newly matured silent synapses do contribute to MPFA measurements after long-term withdrawal from cocaine. However, at this stage, we found a decreased N value, indicating that there are fewer synaptic release points. This seeming discrepancy inspired further investigation into the origin of cocaine-generated silent synapses.

4.4.3 Origin Mechanism of Cocaine-generated Silent Synapses

Based on the observation that the N value decreases after withdrawal from cocaine self-administration but that cocaine-generated silent synapses have disappeared, we attempted to investigate the mechanism of cocaine-induced silent synapse generation. Because silent synapses are thought to be immature synapses, it is natural to assume that they may mature into fully functional synapses. However, there has been much debate over the mechanism by which cocaine generates silent synapses in the NAc. In theory, silent synapses could be formed 1) via the generation of new synapses and the insertion of NMDARs into these new synapses; or 2) via the internalization of AMPARs at existing synapses to leave only NMDARs at these existing

synapses. Much of the debate over the origin mechanism of silent synapses stems from the fact that the methods used to detect silent synapse, such as the minimal stimulation assay, are relative measurements and would provide similar results regardless of the mechanism of generation. Likewise, AMPA/NMDA measurements would also be the same regardless of whether the AMPAR expression decreases to form silent synapses from existing synapses or whether the NMDAR expression increases to form silent synapses at new synaptic contacts.

Additional evidence from our lab and others has largely pointed to the idea that cocaine-generated silent synapses are the result of de novo synapse creation and thus leading to an over-all increase in synaptic NMDARs. This evidence comes from several sources: 1) cocaine-generated silent synapses largely contain NR2B NMDARs, as newer synaptic contacts usually do before being swapped for NR2A NMDARs (Huang et al., 2009); 2) NMDARs composed of newly constructed subunits are incorporated at silent synapses (Huang et al., 2009), 3) AMPAR surface expression largely stays constant during repeated cocaine exposure at times when silent synapses are forming (Boudreau and Wolf, 2005), 4) cocaine increases the frequency of miniature EPSCs in NAc MSNs while PPR does not change (Kourrich et al., 2007), and 5) drugs of abuse including cocaine persistently increase the number of spines and dendritic connections within the NAc (Robinson and Kolb, 2004). Others have suggested that silent synapses may be a byproduct of synaptic scaling processes in the NAc, where AMPARs are removed from MSN synapses after being persistently activated by cocaine to normalize activity within the circuit (Koya et al., 2012). However, there remains much debate, and direct evidence showing the origins of silent synapses has been elusive.

Thus, we sought to determine whether blocking activity-dependent internalization of existing AMPARs at MSNs by over-expression a small peptide that mimics a section of GluA2

C-terminal tail would prevent the appearance of silent synapses in MSNs. By flooding MSNs with this GluA2 C-terminal tail, normal cell signals internalize AMPARs such as PICK1 would instead bind to these freely-available GluA2 C-terminal tail sections and become sequestered before reaching active AMPARs at the membrane (Dong et al., 1997, Kim et al., 2001). Thus, activity-dependent AMPAR internalization should be disrupted, and existing AMPARs should remain at the cell surface. We found that MSNs expressing this GluA2 C-terminal tail segment (3Y variant) during cocaine self-administration did not express increased levels of silent synapses after cocaine self-administration (Fig. 6). MSNs infected with an inactive 3A variant which does not disrupt binding between GluA2 and PICK1, had normal levels of cocaine-generation of silent synapses, as observed in Fig. 2. These results indicate that cocaine generates silent synapses within the PVT-to-NAc pathway by internalizing AMPARs at existing synapses, leaving only NMDARs.

This finding is consistent with the previous data showing a decrease in synaptic release points after withdrawal from cocaine, and may even provide insight into the mechanism of release point reduction. Silent synapses are found at high levels in young and juvenile organisms (Kerchner and Nicoll, 2008, Sametsky et al., 2010), and different patterns of activity can either drive these silent synapses to become strengthened or to be eliminated (Hanse et al., 2013). If existing synapses within the PVT-to-NAc pathway are reverted to the silent state, this could be a first step in a process to eliminate synapses within this pathway. This scenario may explain how cocaine both generates silent synapses and leads to a reduction in the number of release points after long-term withdrawal. Perhaps indicatively, we also observed a trend towards a reduction in the number of synaptic release points after short-term withdrawal from cocaine (from averages of ~20 to ~13, $p = 0.23$), though this reduction did not reach statistical significance.

A significant difference in the levels of silent synapses was also found between 3Y and 3A infected NAc shell cells of saline treated animals, indicating that the internalization of membrane associated AMPARs is necessary even for the maintenance of basal silent synapse levels within the PVT-to-NAc pathway. Given the unique properties of the PVT-to-NAc pathway, however, it is possible that these silent synapses may be generated differently when compared to other pathways. The presence of high basal levels of silent synapses indicates that this pathway may have a high synaptic turnover rate and may be especially sensitive to plasticity-inducing stimuli. Mechanisms may be in place to maintain a certain level of silent synapses within this pathway to preserve a certain metaplasticity. This may be necessarily aided by reverting existing synapses to silent synapses and would be disrupted when internalization of AMPARs is prevented. We did not, however, test other pathways which are known to exhibit cocaine-generation of silent synapses. So, it remains a possibility that silent synapses are generated as new synaptic contacts within other pathways or even, perhaps, via both mechanisms.

4.4.4 Summary

Taken together, it is clear from these results that cocaine self-administration dramatically alters the postsynaptic and presynaptic signaling environment at NAc MSNs for incoming PVT signals. The functional output of NAc MSNs is certainly affected by these signaling changes, especially when considered they are in combination with other cocaine-induced changes in signaling at NAc MSNs. Future investigations into the behavioral correlates of cocaine-driven PVT-to-NAc signaling changes would provide a clearer picture of the importance of these signaling alterations.

5.0 DISCUSSION AND SUMMARY

5.1 OVERVIEW AND INTRODUCTION

We have investigated cocaine-induced signaling changes in 3 distinct systems and found that cocaine affects each system in a discrete manner. We have shown evidence that 1) cocaine exposure increases dynorphin signaling in the NAc, which was also shown to affect excitatory signal strength sent to NAc MSNs; 2) LHb neurons have a greater propensity to fire action potentials after cocaine self-administration, which is correlated with an increase in membrane resistance; and 3) excitatory PVT-to-NAc connections undergo a range of adaptations following cocaine-self administration, including silent synapse-based remodeling and an increase in presynaptic transmitter release. Taken together, these results show complex circuit alterations which affect the flow and transmission of neural information through the mesolimbic system and related areas. It is likely that these described changes contribute to the maladaptive thoughts and behaviors of cocaine addiction.

5.2 DYNORPHIN SIGNALING AND COCAINE

5.2.1 Cocaine-Induced Dynorphin Signaling Changes in the NAc

Activation of kappa or mu opioid receptors in the NAc has previously been shown to acutely decrease EPSCs in MSNs, an effect that appears to be mediated by presynaptic action of the opioid receptor in both cases (Dhawan et al., 1996, Hjelmstad and Fields, 2003). We found that the ability of DynA to reduce EPSCs in NAc shell neurons was completely blocked by the specific kappa opioid receptor antagonist nor-BNI while the ability of DynB to reduce EPSCs was only partially blocked (Mu et al., 2011). We also found that noncontingent cocaine exposure also blocked the ability of kappa opioid receptors and DynA to alter EPSCs. This cocaine-initiated effect could be observed through 2 possible mechanisms: 1) the levels of kappa receptors are dramatically decreased (to this point of disappearing entirely, see Chapter 2, Fig. 3F and 4E) after cocaine or 2) the activation of kappa receptors is now saturated such that further activation attempts at have no effect. Published results related to the effects of cocaine on kappa opioid receptor levels in the NAc are highly inconsistent: no effect (Schroeder et al., 2003, Bailey et al., 2007), upregulation (Hurd and Herkenham, 1993, Unterwald, 2001, Collins et al., 2002), and downregulation (Rosin et al., 1999, Rosin et al., 2000). This variety of results may simply reflect the unstable nature of kappa opioid receptors. However, previous studies have consistently shown that cocaine increases levels of dynorphins in the NAc (Hurd et al., 1992, Spangler et al., 1993, Cole et al., 1995). Due to these considerations, the observed result is most likely due to saturation and/or desensitization of kappa opioid receptors based on an increase in dynorphin signaling in the NAc.

However, DynB's effect on reducing EPSCs is only partially blocked by cocaine or a kappa receptor antagonist. DynB's effects were also not blocked when using a mu opioid receptor antagonist (CTAP) after cocaine exposure. Thus, DynB must reduce NAc MSN EPSCs through kappa and mu receptor-independent mechanisms. DynB is expressed at higher levels in the NAc than DynA (Healy and Meador-Woodruff, 1994), signaling that this unknown mechanism by which DynB reduces NAc EPSCs should still play a significant role in mediating NAc signaling after cocaine exposure. As local electrical stimulation was used in this study to excite all incoming connections to NAc MSNs, we could not discriminate between different pathways. It then remains a possibility that kappa, mu, and other DynB receptors are expressed differently at presynapses based on where the input is coming from and perhaps different cell types. This possibility would cause a general increase in dynorphin signaling by NAc neurons to reduce signaling from some sources of input more than others and bias NAc processing in ways that may contribute to addiction. Considering that a reduction in EPSCs was found after cocaine without a corresponding reduction in IPSCs, this scenario becomes increasingly realistic as it indicates that opioid receptors are specifically not present at inhibitory inputs to the NAc. Further studies are needed to dissect the precise expression of opioid receptors at NAc inputs to clarify this scenario.

5.2.2 Implications of Increased Dynorphin Signaling on NAc Processing

If we assume that dynorphin signaling is broadly upregulated in the NAc after cocaine as suggested by our results and others, and we assume that incoming EPSCs to NAc are broadly inhibited, a situation arises where excitatory input (but not inhibitory input) to NAc MSNs is globally reduced by ~30-50% (Chapter 2, **Fig 1b, e**). This is a large decrease in excitatory drive

that surely has significant effects on NAc function and output. However, other broad changes in excitatory synaptic signaling also occur in the NAc following cocaine exposure. For example, postsynaptic AMPARs are upregulated within a week after cocaine exposure, which could work to counteract the presynaptic reduction in signaling caused by increased opioid receptor activation (Boudreau et al., 2007, Wolf and Ferrario, 2010). Also, the intrinsic excitability of NAc neurons is reduced after exposure to cocaine (Ishikawa et al., 2009, Mu et al., 2010), likely mediated by intracellular homeostatic scaling mechanisms. These adaptations also continue to progress throughout withdrawal periods. Based on the number of changes that occur within the NAc after cocaine, it may be the case that the development of addictive motivations and priorities is an emergent effect resulting from a set of neural adaptations such as these. This is an especially likely scenario when considering the number of studies that demonstrate disruptions in addictive behaviors after disruption of a single signaling system.

5.3 LATERAL HABENULA SIGNALING AND COCAINE

The LHb has been strongly implicated in contributing to motivated behaviors by mediating negative rewards and aversive behavior (Lecourtier and Kelly, 2007, Hikosaka, 2010). We looked at whether LHb neurons underwent any intrinsic signaling changes following cocaine self-administration. Our results showed that LHb neurons were more excitable for at least 7 days during withdrawal from short-access cocaine self-administration, but that the excitability returned to baseline by day 45 of withdrawal (Neumann et al., 2014). The increase in excitability was well correlated with an increase in the membrane resistance, which also returned to baseline by day 45 of withdrawal.

In addition to the results presented in Chapter 3, several pilot experiments were done to measure spontaneous EPSC (sEPSC) activity in LHb neurons from cocaine and saline treated animals. We saw no obvious differences in sEPSCs between these two treatment groups, but sEPCS regularly reached amplitudes upwards of 100 pA (unpublished data). This level of current is similar to the upper current steps measured in our current-clamp recordings. Our data showed individual significant differences at these upper current steps between cocaine and saline groups after ST and MT withdrawal, thus these current steps may also be the most physiologically relevant.

5.3.1 Distinct Cell Populations and Circuits within the LHb

The highly heterogeneous population of LHb cells combined with studies showing projection-specific function and plasticity indicate that various different and perhaps discrete circuits may be present within the LHb (Maroteaux and Mameli, 2012, Good et al., 2013, Jhou et al., 2013, Lecca et al., 2014). This type of heterogeneous organization can make decoding the significance of cellular changes particularly difficult without equally specific methods of discrimination between these circuits. However, several circuit-specific changes have been found in the LHb after cocaine. Cocaine-induced increases in dopamine levels may affect the LHb particularly strongly, due to the high concentration of dopamine transporter proteins in the LHb (Freed et al., 1995). In a subsequent paper published after our initial experiments had been completed, Good et al. (2013) showed that dopamine can elicit excitatory depolarizing currents in LHb neurons in LHb neurons that project to the RMTg but not in LHb neurons that project to the VTA. It was also shown that this dopamine excitation contributes to greater glutamate-mediated excitation in LHb cells and less inhibitory signaling, increasing the overall excitability of LHb cells. An

additional interesting note from this study is that LHb neurons that could be depolarized by dopamine had a more hyperpolarized resting membrane potential, making it possible to identify cells with sensitivity to dopamine depolarization based on their resting cellular characteristics (Good et al., 2013). This information was not known at the time of our experiments and thus was not taken into account, but it would be interesting to see if our observed cocaine-induced changes in membrane excitability and resistance based on average sampling of LHb neurons may actually stem from a similar subpopulation that were averaged together with other neurons, possibly creating a larger effect within a smaller population

5.3.2 Increased LHb Excitability and Addiction

Taken together, the results from the experiments described in Chapter 3 show that LHb neurons are more excitable during a window of time lasting for at least one week after withdrawal from cocaine self-administration. This likely leads to increased LHb transmission during this time, which may be sufficient to trigger additional circuitry changes, such as increased synaptic strength at LHb-RMTg synapses after cocaine exposure (Maroteaux and Mameli, 2012). LHb neurons possess high membrane resistances and are highly sensitive. The observed changes in excitability levels are more like to affect *how many* action potentials an LHb neuron fires in response to incoming excitatory input, rather than *if* an LHb will fire. This change may lead to an increase in excitatory signaling sent to the RMTg, which in turn would cause RMTg neurons to increase inhibitory signals sent to VTA dopamine neurons.

In addition to this increased inhibitory effect in the VTA via the RMTg, recent evidence also shows that a large portion of LHb neurons which project directly to the VTA are inhibited by dopamine (~50%, via D_2 receptors) (Good et al., 2013). However, it appears that the direct

LHb-to-VTA projection targets GABAergic neurons in the VTA (Stamatakis and Stuber, 2012), and that these VTA neurons are part of a subpopulation which project to the mPFC (Lammel et al., 2012). It is currently unclear how this pattern of signaling may contribute to processing of motivated behaviors.

Nevertheless, stimulation of LHb neurons causes a strong transient inhibition in VTA dopamine neurons (Ji and Shepard, 2007). An increase in inhibitory signaling to VTA dopamine neurons via increase LHb signaling would be consistent with the observed role of the LHb in mediating negative rewards and aversive behaviors (Matsumoto and Hikosaka, 2009, Hikosaka, 2010, Lammel et al., 2012, Good et al., 2013, Jhou et al., 2013, Lecca et al., 2014), and it may contribute to cocaine-induced increases in negative affect that drives persistent drug-seeking behavior (Solomon and Corbit, 1974, Solomon, 1980, Koob et al., 2014). Further work is needed to better understand these complex neural circuits and how changes to their neural signaling may lead to addiction.

5.4 COCAINE-INDUCED CHANGES IN SIGNALING BETWEEN THE PARAVENTRICULAR NUCLEUS OF THE THALAMUS AND THE NUCLEUS ACCUMBENS

5.4.1 Generation of Silent Synapses and Implications of High Silent Synapses Levels

Analysis based on the structure and anatomy of NAc shell MSNs led to suggestions that these neurons may have a specific propensity to undergo plasticity via the creation of new spines and synapses rather than traditional LTP/LTD processes (Meredith et al., 2008). It was then demonstrated that cocaine induced silent synapses in randomly sampled NAc neurons (Huang et al., 2009, Brown et al., 2011) and also specifically within certain pathways to the NAc (Lee et al., 2013, Ma et al., 2014). Our results, presented in Chapter 4, show that the PVT-to-NAc shell pathway contains a relatively high baseline level of silent synapses, and that additional silent synapses are generated in response to cocaine self-administration.

Silent synapses are intriguing synaptic structures, as it appears that the generation of silent synapses acts as a form of metaplasticity (Lee and Dong, 2011). Silent synapses can either be strengthened into functional synapses by inserting AMPARs at the membrane or they can be atrophied and removed (Isaac et al., 1995, Hanse et al., 2013). Thus, the presence of high levels of silent synapse within connections from the PVT to the NAc implies that this pathway has a high propensity for plasticity. Because the PVT may be preferentially activated by conditioned drug rewards and reward cues rather than appetitive stimuli (Brown et al., 1992, Wedzony et al., 2003, Matzeu et al., 2014), it may be the case that high silent synapse levels within this pathway allow for rapid learning and conditioning of new and/or novel stimuli. The rewarding effects of

cocaine and other drugs may become quickly engrained via this pathway as new motivational drives.

An intriguing hypothesis to consider, then, is that cocaine may dramatically reorganize the PVT-to-NAc pathway to bias PVT transmission to correspond to motivation for cocaine rewards while also weakening the ability of the PVT to send signals related to motivation for other types of rewards, such as natural rewards. Our evidence shows that there may be reduced synapse numbers in the PVT-to-NAc pathway after long-term withdrawal from cocaine self-administration, but that the remaining synapses have stronger signal transmission. If the fewer remaining synaptic connections correspond specifically to conditioned cocaine cues and rewards while the eliminated connections corresponded to other types of conditioned rewards, the circuit would be strongly biased towards cocaine-associated output and biased against other cues or conditioned stimuli. Many different pathways and signals contribute to motivated behaviors, but the repurposing of the PVT-to-NAc pathway to promote signaling based on cocaine cues while simultaneously reducing the influence of other conditioned cues in this way could be a particularly strong neural influence on NAc signaling and could contribute to addictive behavior (Kelley and Berridge, 2002). This may also help to explain why motivations to seek addictive drugs come to outweigh motivation for other types of reward during addiction. One recently described study in support of this hypothesis contends that conditioned cocaine rewards preferentially activate PVT neurons relative to conditioned appetitive rewards (Martin-Fardon et al., 2013, Matzeu et al., 2014). Another study has shown that the PVT preferentially activates in response to conditioned ethanol rather than conditioned sucrose (Wedzony et al., 2003). These studies suggest that the PVT may become especially responsive to drug-related stimuli after

prolonged withdrawal over appetitive-related stimuli. Our results in Chapter 4 and these studies would indicate that this hypothesis warrants further investigation.

5.4.2 Maturation of Silent Synapses

Our lab has shown that the maturation of cocaine-generated silent synapses correspond to incubation of cocaine craving throughout withdrawal via the insertion of CP-AMPARs at maturing silent synapses after long-term withdrawal (Lee et al., 2013, Ma et al., 2014). Importantly, while pathways that attenuate cocaine-seeking behavior (prelimbic PFC-to-NAc pathway) also generate silent synapses after cocaine exposure, these synapses did not recruit CP-AMPARs during maturation. Thus, the appearance of silent synapses and the subsequent recruitment of CP-AMPARs to these NAc shell synapses appear to be critical for cocaine-induced molecular changes to NAc signaling that influence craving and relapse behavior.

CP-AMPARs were also found at PVT-to-NAc synapses in cocaine-naïve rats, but CP-AMPAR levels did not change after long-term withdrawal from cocaine. Based on results from our lab's previous studies (Lee et al., 2013, Ma et al., 2014), this may indicate that the PVT-to-NAc pathway is not involved in incubating cocaine craving during withdrawal. However, the role of CP-AMPARs within the PVT-to-NAc pathway remains perplexing. CP-AMPARs only account for ~5% of all AMPARs in the NAc under control conditions (Reimers et al., 2011). Yet, we see that CP-AMPARs account for ~20% of AMPAR-mediated synaptic current along the PVT-to-NAc pathway. CP-AMPARs have a higher conductance than other AMPARs (Liu and Zukin, 2007), so the actual basal expression rate of CP-AMPARs is likely slightly less than ~20%. However, their role and their effect on PVT-to-NAc signaling remain unclear.

One reason why an increase in CP-AMPARs after long-term withdrawal may not have been observed might be due to the cocaine self-administration procedure that was used. There are conflicting reports concerning the appearance of CP-AMPARs after cocaine exposure. One lab reports failing to observe CP-AMPARs after using a short-term (2 hour) daily self-administration protocol and have reported that a longer training period involving greater daily exposure to cocaine is needed (Purgianto et al., 2013). However, our lab has had success inducing CP-AMPAR expression after long-term withdrawal when using a 2-hour daily self-administration protocol (Lee et al., 2013). Perhaps CP-AMPARs appear more readily within certain pathways relative to others.

5.4.3 Orexin Signaling in the PVT

Orexin is an excitatory signaling peptide produced exclusively in the hypothalamus, and then transmitted to regions throughout the brain including the PVT, VTA, and NAc shell (Peyron et al., 1998, Kirouac et al., 2005). Intracerebroventricular injections of orexin reinstate cocaine seeking behavior (Boutrel et al., 2005), and orexin neurons in the lateral hypothalamus become activated by exposure to stimuli associated with cocaine (Harris et al., 2005). Orexin neurons do play a role in mediating natural reward processes such as food (Harris et al., 2005, Kelley et al., 2005, Choi et al., 2012). However, a report indicates that orexin receptor antagonists may preferentially block conditioned cocaine seeking (Martin-Fardon and Weiss, 2014) and ethanol seeking (Jupp et al., 2011) while having no effect on appetitive reward seeking. Thus, the orexin system may be preferentially involved in drug reward processing over appetitive reward processing. This type of effect may be mediated via orexin receptors and neural activity in the PVT, as a similar bias for conditioned cocaine- and ethanol-motivations over conditioned

appetitive reward processes may also exist within the PVT (Wedzony et al., 2003, Martin-Fardon et al., 2013). This pattern seems to further indicate that orexin and PVT signaling may be important in neural processes that distinguish drug-related motivations from other types of natural rewards. This activity may have crucial implications for the development and maintenance of addictive states and should be investigated further.

An additional point of note is that another neuropeptide named 'Cocaine- and amphetamine-regulated transcript' (CART) appears to target the PVT and is also largely produced in the hypothalamus (Kirouac et al., 2006). Contrary to orexin's excitatory effects, signaling activity via this peptide to the PVT is correlated with reduced cocaine-primed reinstatement (James et al., 2010). Thus, hypothalamus-to-PVT signaling may have bidirectional control in regulating motivation for cocaine-seeking-related behavior via orexin and CART signaling. This activity is presumably mediated by regulating the excitation levels of PVT neurons, which then project to the NAc and other addiction-related regions.

5.4.4 PVT Interaction with Dopamine in the NAc

PVT-to-NAc fibers can synapse adjacent to dopamine fibers at NAc neurons (Pinto et al., 2003). Stimulation of the PVT also induces dopamine release within the NAc (Jones et al., 1989, Parsons et al., 2007). This neural architecture leads to an additional layer of complexity in PVT-to-NAc signaling. If PVT inputs are adjacent to postsynaptic D2 receptors, then dopamine signaling would counteract excitatory PVT signals. But, if PVT synapses are adjacent to D1 receptors, dopamine signaling would complement excitatory PVT signals to NAc MSNs. PVT projections to D1 receptor-expressing MSNs may be well-positioned to be especially strengthened in response to drug-related stimuli. Increased excitatory modulation via dopamine

activation of D1 receptors would directly potentiate PVT glutamatergic signals to these NAc MSNs. Thus, addictive drugs may be especially efficient in promoting the strength of PVT-to-NAc connections by increasing dopamine signaling, and this processes may also help to strengthen these connections specifically. However, the expression pattern of D1 and D2 receptors in the NAc is scattered without clear organization and has yet to be examined with respect to PVT inputs.

5.4.5 Dynorphin Signaling and Opioid Receptor Expression in the NAc

In section 5.1.1 above, we speculated that dynorphin signaling may affect certain pathways to the NAc but not others based on selective expression of opioid receptors at some inputs. We showed that dynorphin signaling is likely increased in the NAc after cocaine, leading to a presynaptic reduction of EPSCs in NAc MSNs. However, we also showed the probability of presynaptic release specifically in the PVT-to-NAc pathway is increased following cocaine exposure at the same time points when dynorphin signaling was measured to reduce presynaptic release. Dynorphin-mediated reduction in EPSCs could instead be due to a reduction in quantal size rather than presynaptic release rate. However, we also did not observe a change in quantal size after short-term withdrawal within the PVT-to-NAc pathway at these time points. Thus, it appears unlikely that PVT-to-NAc synapses are affected by dynorphin signaling and may not express presynaptic opioid receptors. This interpretation makes it increasingly likely that the expression of opioid receptors is selective to certain NAc inputs and that increases in dynorphin signaling following cocaine exposure may influence a subset of NAc inputs.

5.4.6 Multiple Probability Fluctuation Analysis Technique Discussion

Consistent with the presence of CP-AMPARs, we saw a paired-pulse facilitation effect at PVT-to-NAc synapses. Synapses containing CP-AMPARs often demonstrate paired-pulse facilitation because CP-AMPARs can be blocked by intracellular polyamines, but this block is displaced by rapid successive stimulations (Rozov and Burnashev, 1999). The decrease in paired-pulse facilitation after cocaine self-administration is most likely related to the increase in presynaptic release probability rather than functional expression of CP-AMPARS because pharmacological block of CP-AMPARs showed no change in their contribution to EPSCs following cocaine.

PVT-to-NAc synapses showed changes in PPR following cocaine self-administration, which indicates that presynaptic changes occurred following cocaine-self administration. We investigated the specifics of these presynaptic changes using MPFA and found that PVT-to-NAc synapses have a higher Pr after cocaine. After long-term withdrawal, we found that the increased Pr persisted and also that there were fewer presynaptic release points with stronger transmission based on having a larger quantal size. Presynaptic release points could potentially correspond to extra-synaptic glutamate release sites or other non-traditional release formations, but this measure was largely interpreted as reflecting functional synapses. Silent synapses are thus excluded because they conduct no appreciable EPSCs which are used in MPFA measurements. Thus, the number of PVT-to-NAc synapses is thought to be reduced after long-term withdrawal from cocaine self-administration, but the strength of these synapses is thought to be higher based on higher Pr and larger quantal content.

Roughly 30% of cells were excluded from analysis with MPFA because a binomial curve could not be well fit to the variance-mean relationship of the 5-pulse stimulation train. This

indicates that this section of cells did not meet the assumptions of the MPFA model: (1) Pr is uniform across release sites, (2) release sites operate independently, (3) release is synchronous, and (4) Q is uniform at an individual site and across release sites (Silver, 2003, Suska et al., 2013). It's unclear why exactly this section of PVT-to-NAc connections which did not meet these assumptions, but we think it is most likely related to point 1 or 4. A change in the probability of release or the quantal size across synapses may relate to differing inputs. Because we observed changes in both Pr and Q after cocaine, these properties appear to flexible properties within this pathway. Thus, if Pr or Q values varied too greatly based on differing inputs, the variance-mean analysis would not be fit to a binomial curve. Points 2 and 3 are assumed to be relatively stable in slice preparations such as those used in our experiments.

Another caveat of the MPFA technique is that making comparisons based on the number of presynaptic release points between cells can be highly variable. This value corresponds to the number of release sites which are activated by the stimulation used to generate the 5-pulse data. So, there are regularly some relevant synapses that are not included in this analysis, as all connecting synapses are unlikely to be stimulated. When using optogenetic stimulation, such as was done in our experiments, this value is also dependent on the expression rate of the opsin channel in eligible projecting fibers. The opsin plasmid does not infect every projecting cell, and thus an additional proportion of projecting fibers are invariably left out from analysis when using optical stimulation. However, for our experiments, there does not appear to be any bias in infection rates between treatment groups because the same virus and procedure was used to infect PVT neurons in both groups. No differences in infection rate were obvious between the groups. Additionally, the same optical stimulation intensity and duration (1.5 ms) was used for most all experiments, reducing the number of variables which could contribute to differences in

the number of presynaptic release sites activated between subjects. Thus, the N values calculated from MPFA are unlikely to be accurate as raw values for the total number of PVT-to-NAc synapses (because some portion of synapses are not included when collecting the data), but the N values are accurate as relative measurements between groups under our controlled conditions.

5.4.7 Blocking AMPAR Internalization Blocks Cocaine-generation of Silent Synapses in the PVT-to-NAc pathway

Because we observed an increase in silent synapses within the PVT-to-NAc pathway after cocaine self-administration but our MPFA data indicated that fewer synaptic connections existed after long-term withdrawal, we wanted to explore this relationship further. Silent synapses can potentially be formed by creating new synaptic connections with inserted NMDARs or from existing synaptic connections by internalizing AMPARs. We used a virally mediated plasmid to express a peptide mimicking the C-terminal tail of the AMPAR GluA2 subunit. The C-terminal tail of GluA2 contains a PDZ-binding domain which interacts with PICK1 to signal internalization of AMPARs (Dong et al., 1997, Kim et al., 2001). By expressing this peptide sequence, these internalization protein signals would bind to the peptide instead of functional AMPARs at the synapse, effectively sequestering these internalization signals (Kim et al., 2001).

We found that expression of this GluA2 C-terminal tail peptide reduced both cocaine-generation of silent synapses and basal levels of silent synapses in infected neurons. Expression of an inactive control variant of this peptide had no effect on silent synapse levels. This result indicates that silent synapses are formed within the PVT-to-NAc pathway via internalization of AMPARs at existing synapses. When AMPARs internalization signals are blocked, silent synapse generation is thus also blocked. This result seems to fit well with our other data showing

that there may be a reduction in synapse numbers after long-term withdrawal from cocaine. Reverting existing functional synapses to a silent state may be the first step in eliminating these synapses entirely (Hanse et al., 2013). Previous studies in our lab have indicated that cocaine-generate silent synapses mature by recruiting AMPARs to become functional synapses (Huang et al., 2009, Brown et al., 2011, Lee et al., 2013, Ma et al., 2014). But, the high basal level of silent synapses and the basal presence of CP-AMPARs in the PVT-to-NAc pathway may indicate that this pathway has unique properties related to plasticity. The lack of CP-AMPAR recruitment to silent synapses after long-term withdrawal from cocaine may also indicate these synapses do not mature normally or similarly to other pathways.

GluA1/2 AMPARs comprise about 81% of synaptic AMPARs in the NAc, while GluA2/3 AMPARs make up about 16% (Lu et al., 2009). GluA1/2 AMPARs have longer C-terminus tails and are trafficked to and from synapses in an activity-dependent manner, while GluA2/3 AMPARs have short C-terminus tails and cycle in and out of synapses constitutively (Malinow, 2003). The GluA2 tail peptide blocks PICK1 internalization interactions, which is thought to be specific for GluA1/2 AMPARs which moved to and from synapses in an activity-dependent manner, and constitutive cycling of GluA2/3 AMPARs is thought to be preserved (Kim et al., 2001, Malinow, 2003). Based on this functionality, the 3Y virus thus also blocks activity-dependent LTD-like processes that involve the internalization of AMPARs. It is possible that this inability to initiate LTD processes in infected NAc MSNs (rather than direct internalization of AMPARs) is responsible for the lack of observed silent synapses after cocaine exposure. Possible indirect effects that blocked LTP has on the formation of silent synapses is unclear, but the most straight forward interpretation of our current results is that AMPAR internalization is required for the formation of silent synapses within the PVT-to-NAc pathway.

5.5 CONCLUDING REMARKS

Cocaine exposure very likely leads to cascades of cellular changes which lead to the development of maladaptive thoughts and behaviors. It is critical to identify and decode these cellular changes in order to understand the mechanisms of addiction and to find possible treatment targets. The most clinically relevant treatments for addiction are all related to circumstances when addictive thoughts and behaviors have already established themselves. It is therefore important to understand the cellular and molecular changes that underlie these thoughts and processes so that realistic and clinically relevant treatments can be found.

Taken together, the results detailed here demonstrate a number of previously unknown cocaine-induced neural adaptations in regions related to motivational processing and addictive behavior. Major targets for further research are identified, including PVT neural signaling to the NAc (potentially related to orexins and CART) which may be related to the development of novel drives and motivated behaviors, and LHb and dynorphin signaling which may be related to the aversive effects of cocaine which likely contribute to relapse during withdrawal periods. Hopefully, future studies can further expand on the results presented here and build on them to construct a better understanding of the array of neural changes responsible for addiction.

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