

Characterization of GABAergic projection from the ventral tegmental area to the nucleus accumbens: Effects of dopamine and cocaine

by

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Bachelor of Science, Neuroscience, Washington State University, 2007

Submitted to the Graduate Faculty of
the Kenneth P. Dietrich School of Arts & Sciences in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2015

UNIVERSITY OF PITTSBURGH
KENNETH P. DIETRICH SCHOOL OF ARTS & SCIENCES

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**CHARACTERIZATION OF GABAERGIC PROJECTION FROM THE VENTRAL
TEGMENTAL AREA TO THE NUCLEUS ACCUMBENS: EFFECTS OF
DOPAMINE AND COCAINE**

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Within the mesolimbic dopamine (DA) system, the VTA-to-NAc projection is essential for many emotional and motivational responses, and is often targeted by drugs of abuse to produce cellular and behavioral alterations. Whereas the DAergic component in this projection has been extensively examined for its role in mediating the primary reward effect and reward prediction, this projection also contains a significant GABAergic component and a relatively weak glutamatergic component. These two fast projections are often concurrently activated with the DAergic projection upon incentive stimuli, but their physiological properties and their relationship with the DAergic component have not been well understood. Focusing on this knowledge gap, our present study utilized a multidisciplinary approach combining electrophysiology, genetics/optogenetics, and operant behavioral tests, to characterize VTA-to-NAc GABAergic projection, its interaction with parallel DAergic projection, and its adaptive changes after cocaine exposure. The parallel glutamatergic projection was examined for comparison. Our results showed that VTA-to-NAc GABAergic transmission exhibited relatively high presynaptic release probability and fast-decaying postsynaptic responses. Activation of parallel DAergic projection induced LTD at VTA-to-NAc GABAergic synapses heterosynaptically. After withdrawal from repeated cocaine exposure, we detected several profound alterations at VTA-to-NAc GABAergic

synapses, among which the D1-dependent heterosynaptic LTD was abolished. Furthermore, cocaine-induced alterations in GABAergic and glutamatergic transmissions are highly dynamic, critically depending on cocaine procedures and drug withdrawal periods. These results reveal that GABAergic and glutamatergic projections exert direct inhibitory and excitatory effects on NAc principal neurons, and the shifted inhibitory-excitatory balance in NAc neurons may contribute to the shifted functional output of the NAc. Moreover, given the prominent regulatory role of DA in emotional and motivational response, loss of DA-dependent heterosynaptic plasticity at VTA-to-NAc GABAergic synapses during cocaine withdrawal echoes several withdrawal symptoms such as rigid emotional state favorable to drug taking, reduced responses to non-drug-related emotional stimuli, and persistent hypoactivity of the NAc. The novel roles of VTA-to-NAc GABAergic projection demonstrated in the present study may pave the road for future studies to understand the circuitry-based mechanisms of physiological and pathophysiological emotional and motivational responses.

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PREFACE

Acknowledgements

I would like to express my great appreciation to those who have supported my graduate education in both Washington State University and the University of Pittsburgh. First, I thank my mentor, Dr. Yan Dong, for his supervision, guidance, supports, and patience. He has devoted countless hours to my projects and career development, without which I could not reach where I am. I would also thank my training supervisor, Dr. Masago Ishikawa, who taught me electrophysiology from the scratch. I shared many projects with him, and enjoyed sitting together with him discussing projects, career, and opportunities. I would also like to thank my lab mates, especially Drs. Brian Lee, Bradley Winters, Anna Suska, Peter Neumann, and Yanhua Huang, for their friendship and assistance. I would also like to express my sincere gratitude to my dissertation committee members, Drs. Alan Sved, Tony Grace, Susan Sesack, and Aryn Gittis for their professional advices and guidance. I would also like to thank Dr. Marina Wolf for joining my dissertation committee as the external member. The training opportunities I received from my committee members were so precious to me. In addition, I am taking this opportunity to express my gratitude to the staff members who support my research and education, especially Joan Blaney. Not only the guidance for the paperwork but also the undiminished encouragement she has provided helped me in many occasions throughout my life in Pitt. I also thank my friends and professors in the Department of Integrative Physiology and Neuroscience at Washington State University. Even after the move to Pittsburgh, they are being good friends to me and the conversations with them always cheered me up. Last but not least, I thank my families and best friends. I believe I am lucky to have great people both in my home country and in the US. I thank

my “mother” and “sisters” in America, Jodi, Marin, and Mikino Ishikawa, for their kindness and cheerfulness. I really appreciate the supports and encouragements from my beloved parents, Yuko and Tetsunori Otaka. They gave me the chance to see a different world and to learn many things through the life in the United States.

List of Abbreviations

AAV	Adeno-associated viral vector
aCSF	artificial cerebrospinal fluid
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BLA	Basolateral amygdala
CaMK	Calcium-calmodulin kinase cyclic
cAMP	Cyclic adenosine monophosphate
ChR2	Channelrhodopsin 2
CIN	Cholinergic interneuron
CREB	cAMP-response element-binding protein
DA	Dopamine
DAT	Dopamine transporter
eCB	Endocannabinoid
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
FSIs	Fast-spiking interneurons
GABA	γ -Aminobutyric acid
iLTP	Inhibitory Long-term potentiation
i.p.	Intraperitoneal
IPI	Interpulse interval
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
KCC2	Potassium-chloride- co-transporter

LTD	Long-term depression
LTP	Long-term potentiation
MSN	Medium spiny neuron
NAc	Nucleus Accumbens
NAcCo	Nucleus Accumbens Core
NAcSh	Nucleus Accumbens Shell
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NKCC1	Sodium-potassium-2 chloride co-transporter
NMDA	N-methyl-D-aspartate
PBP	Parabrachial pigmented area
PFC	Prefrontal Cortex
PN	Paranigral nucleus
PPR	Paired-pulse ratio
PTX	Picrotoxin
SNc	Substantia nigra pars compacta
TEA-Cl	Tetraethylammonium chloride
TH	Tyrosine hydroxylase
TTX	Tetrodotoxin
VGLUT2	Vesicular glutamate transporter 2
VP	Ventral Pallidum
VTA	Ventral Tegmental Area

1.0 INTRODUCTION

1.1 DRUG ADDICTION

Drug addiction is a chronic brain disease that leads to harmful behaviors; it is characterized by abnormally increased “motivation” to take drugs and the reduced ability to control drug taking behavior (Kalivas & Volkow, 2005). Motivation is ever-present and an essential determinant of behaviors and adaptations for all animals. It could be simple and short-term just for survival, such as obtaining food and water, and also could be social and long lasting. Because it is critical for the survival of animals, the abnormality in motivation causes serious medical and social problems. In the case of drug addiction, a report from the National Institute on Drug Abuse estimates that drug abuse costs Americans half a trillion dollars a year to take care of medical, economic, criminal and social impacts of drug addiction. In addition, the report mentions that more than 100,000 people die from drug addiction related causes in the United States each year (National Institutes on Drug Abuse, 2010). Thus far, there are few effective treatments available for drug addiction. This urgent unmet medical need calls for extensive efforts to understand the molecular and cellular mechanisms underlying drug addiction and related motivational disorders.

1.2 ANIMAL MODELS

Animal models, such as the drug self-administration model, are used to understand drug-induced behaviors and the brain circuits that underlie these behaviors. Self-administration of drugs of abuse is an operantly conditioned response in which the reward is the drug. The self-administration model is considered to have high validity for studying drug-seeking behaviors (Shaham & Hope, 2005). In this model, the animals are implanted with an intravenous catheter and are trained on the task, which usually involves pressing a lever or poking their nose into a hole to obtain the reward (a drug in this case). A wide variety of manipulations is possible in self-administration, and different cues can be associated with drug self-administration (Panlilio & Goldberg, 2007), which makes this model similar to human conditions in which drug-associated cues in the environment produce relapse (Collins, Weeks, Cooper, Good, & Russell, 1984).

1.3 BRAIN CIRCUITS INVOLVED IN DRUGS OF ABUSE

The motivational drive and behavioral output are regulated by the brain reward systems, among which the mesocorticolimbic dopamine (DA) system has been shown to mediate a variety of emotional and motivational disorders, including drug addiction (Hyman, Malenka, & Nestler, 2006; Wise, 1996). The mesolimbic DA system involves DA cell bodies in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc) and other structures, such as the prefrontal cortex (PFC), amygdala, and hippocampus (Kauer & Malenka, 2007). All addictive substances cause an increase in DA concentration in the projection areas of the VTA and the VTA itself (Lüscher & Malenka, 2011). However, the mechanisms that drugs of abuse increase DA

concentrations are different. Psychostimulants, such as cocaine and amphetamine, target dopamine transporters (DATs), resulting in DA accumulation.

The NAc receives inputs not only from the VTA but also other brain regions, and those inputs include excitatory, inhibitory, and modulatory inputs. Integration of those excitatory/inhibitory inputs with DAergic modulation is important for motivational/emotional behaviors. However, addictive drugs destroy this well-organized system, and it leads the abnormal behaviors (Sesack & Grace, 2010).

1.3.1 Nucleus Accumbens

The NAc was recognized as one of the “pleasure centers” of the brain in the 1950s. Stimulation of the area using implanted electrodes produced rewarding effects (Olds & Milner, 1954). Subsequent work conceptualizes the NAc as an interface between emotion/motivation and behavior (Mogenson, Jones, & Yim, 1980). Since then, the NAc has been studied extensively for its roles in reward-elicited behaviors.

The NAc comprises two main anatomical components, the NAc shell (NAcSh) and the NAc core (NAcCo) (Brog, Salyapongse, Deutch, & Zahm, 1993; Zahm & Brog, 1992). Inputs from different brain regions to the NAc differently innervate NAcSh and NAcCo neurons (Brog et al., 1993). For example, the NAcSh receives inputs preferentially from the infralimbic PFC, the ventral prelimbic PFC, the ventral agranular insular cortex, the orbital cortex, the ventral hippocampus, and the caudal part of BLA. On the other hand, the NAcCo receives inputs mainly from the dorsal prelimbic cortex, the dorsal agranular insular cortex, and the rostral part of BLA (Britt et al., 2012; Brog et al., 1993; Sesack & Grace, 2010; Zahm, 2000). Each excitatory input from different regions shows distinct physiological and behavioral roles (Sesack & Grace, 2010).

In a simplified view, the NAcSh is considered as a transitional location between the striatum and the extended amygdala and may be involved in the rewarding effects, while the NAcCo is considered as an extension of the dorsal striatum and may be important for instrumental learning, including cue-conditioned drug seeking behavior (Kauer & Malenka, 2007; Kelley, 2004; Sesack & Grace, 2010).

The majority of neurons in the NAc are medium spiny neurons (MSNs), which are GABAergic (Meredith, 1999). There are two major subtypes of MSNs, one expressing DA D1 receptor and the other expressing DA D2 receptors. In the dorsal striatum, D1-expressing MSNs are dominated in the direct pathway projection and D2-expressing MSNs are dominated in the indirect pathway projection (Sesack & Grace, 2010). In addition, electrophysiological results suggest that many striatal MSNs co-express both D1 and D2 receptors. NAc MSNs can also be roughly divided as D1 and D2 receptor-expressing neurons. Whereas a general opinion is that D2-expressing NAc MSNs preferentially project to the ventral pallidum (VP) and D1-expressing NAc MSNs project to the VP and the VTA (K.-W. Lee et al., 2006; Lu, Ghasemzadeh, & Kalivas, 1998), the actual projection patterns are not as clear-cut as in the striatum (Stefanik, Kupchik, Brown, & Kalivas, 2013).

Lacking internal pace-making mechanisms, the functional output of NAc MSNs (i.e., action potential firing) is heavily influenced by fast synaptic inputs. The glutamatergic afferents play important roles for NAc output. In addition to fast excitatory inputs, the NAc receives fast inhibitory synaptic inputs (Gustafson, Gireesh-Dharmaraj, Czubyko, Blackwell, & Plenz, 2006; Tepper, Koos, & Wilson, 2004). Local interneurons, including several types of GABAergic interneurons and cholinergic interneurons (CINs), in the NAc play important roles in the timing and synchronization of NAc output (Berke, Okatan, Skurski, & Eichenbaum, 2004; Berlanga et

al., 2003; Gruber, Powell, & O'Donnell, 2009; Koos & Tepper, 1999; Lansink, Goltstein, Lankelma, & Pennartz, 2010; Tepper & Bolam, 2004). Also, NAc MSNs receive inputs from axon collaterals from neighboring MSNs (Faure, Richard, & Berridge, 2010). Moreover, the NAc receives inhibitory inputs from the VP and the VTA (Sesack & Grace, 2010). These inhibitory synaptic inputs make NAc MSNs resistant to excitation, while synchronous excitatory synaptic inputs drive NAc MSNs into the active state (Gambrill & Barria, 2011; Graybiel, 1998; O'Donnell & Grace, 1995; Wilson & Kawaguchi, 1996; Wilson, 1986).

1.3.2 Ventral Tegmental Area

The VTA is located around the midline on the floor of the midbrain. The VTA is comprised of a heterogeneous population of cells. The VTA contains DAergic (~65%), GABAergic (~30%), and glutamatergic (~5%) neurons that project both within and outside of the VTA, such as the NAc and the PFC (Carr & Sesack, 2000; Dobi, Margolis, Wang, Harvey, & Morales, 2010; Johnson & North, 1992b; Nair-Roberts et al., 2008; Yamaguchi, Sheen, & Morales, 2007). Although GABAergic neurons in the VTA have been often described as interneurons, increasing evidence suggests that some of VTA GABAergic neurons project in long-range, parallel to DAergic neurons (Carr & Sesack, 2000; Steffensen, Svingos, Pickel, & Henriksen, 1998). In addition, several studies indicate that some DAergic neurons may co-release glutamate or GABA (Stuber, Hnasko, Britt, Edwards, & Bonci, 2010; Sulzer et al., 1998; Tritsch, Ding, & Sabatini, 2012).

DA release from the VTA appears to be necessary for the primary rewarding effects of drugs of abuse and regulates positive reinforcement of drug administration (Di Chiara & Imperato, 1988; Di Chiara, 1998; Schultz, 2006). Drugs of abuse are powerful triggers of synaptic plasticity in the VTA (Jones & Bonci, 2005; Lüscher & Malenka, 2011). Also, the plasticity in the VTA

triggers the long-term cellular adaptations in the downstream brain regions, including the NAc (Kauer & Malenka, 2007).

Although there is strong evidence supporting the reward prediction error hypothesis (Schultz, 1997), DAergic neurons also play a role in aversive events. A recent study detects diverse responses of DAergic neurons in and around the substantia nigra pars compacta (SNc) and the VTA. Some studies found that the aversive events excite midbrain DAergic neurons, whereas others report suggest VTA DAergic neurons are inhibited by aversive events (Lammel, Lim, & Malenka, 2014; Matsumoto & Hikosaka, 2009). In addition, another study indicates that the increased activity of DAergic neurons in the aversive event may signal the termination of the aversive stimulus as rewarding (Lammel et al., 2014; Tanimoto, Heisenberg, & Gerber, 2004). Most VTA DAergic neurons, which show inhibitory response by an aversive event, also show phasic excitation when the aversive event is terminated (Brischoux, Chakraborty, Brierley, & Ungless, 2009). Classically, it has been considered that DAergic neurons are inhibited by aversive events (Schultz, 1997). Following this idea, a later study indicates that the increased activity of DAergic neurons in aversive events may be considered as a reward because of the termination of aversive events (Tanimoto et al., 2004). However, there is evidence exhibiting that DAergic neurons located in a specific region of the VTA show excitation during the onset of aversive event (Brischoux et al., 2009). Together with those findings, increasing evidence suggests the large heterogeneity of DAergic neurons. A study indicates the possibility that the excited VTA neurons by aversive stimuli may not be DAergic neurons (Ungless, Magill, & Bolam, 2004). A recent study demonstrates that most VTA GABAergic neurons are excited by aversive events (Cohen, Haesler, Vong, Lowell, & Uchida, 2012).

In addition, studies show that addictive drugs impair the VTA GABAergic transmission to VTA DAergic neurons (Liu, Pu, & Poo, 2005; Lüscher & Malenka, 2011). The impaired transmission leads increased DA release and may be a possible mechanism of the reinforcing effect of drugs of abuse (Hyman et al., 2006; Johnson & North, 1992a; Lüscher & Malenka, 2011; Tan et al., 2010; Vashchinkina, Panhelainen, Aitta-aho, & Korpi, 2014).

1.4 EXCITATORY TRANSMISSION

NAC MSNs receive glutamatergic synaptic inputs from the PFC, hippocampus, amygdala, thalamus, VTA, and other brain regions. MSNs in the NAc integrate information from those regions, and through its projections, regulate motivational behaviors (Wolf, 2010). α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate receptor (NMDAR) are the main postsynaptic ionotropic receptors at glutamatergic synapses that mediate fast excitatory synaptic transmission.

1.4.1 NMDAR

The NMDAR is composed of three common subunits; GluN1, GluN2 and GluN3 (Cull-Candy, Brickley, & Farrant, 2001). The most unique characteristic of NMDAR that makes it different from other ligand-gated ion channels is the voltage-dependent magnesium block, which can be removed after depolarization (Purves et al., 2008; Traynelis et al., 2010). The permeability for calcium ions is another important property because Ca^{2+} triggers intracellular signaling cascades, such as calcium-calmodulin kinase (CaMK) signaling (Malenka & Nicoll, 1999) and

activation of transcription factors, such as cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB). The composition of NMDARs determines the magnitude of NMDAR-mediated synaptic currents and the permeability to calcium (Lau & Zukin, 2007). GluN2 subunits play a critical role for the plasticity at pharmacological and electrophysiological aspects. The slow decay kinetics of NMDARs allows post-synaptic membrane for wide summation window for excitatory postsynaptic potentials (EPSPs). GluN2A- and GluN2B-containing NMDARs possess different decay kinetics and binding affinity to calcium signaling proteins. The ratio of GluN2B/2A subunits decreases as animals age (Cull-Candy et al., 2001). These different properties may render GluN2A- and N2B-containing NMDARs with different roles in regulating long-term potentiation (LTP) and long-term depression (LTD), and other forms of synaptic plasticity (Dong & Nestler, 2014; Shipton & Paulsen, 2014).

1.4.2 AMPAR

AMPARs, which open and close faster than NMDARs, contribute most fast synaptic transmission at glutamatergic synapses (Platt, 2007). There are four common AMPAR subunits, GluA1, GluA2, GluA3, and GluA4, which, via different combinations, can form a tetrameric AMPAR (Malinow & Malenka, 2002; Song & Huganir, 2002). In NAc MSNs of drug-naive rodents, most AMPARs contains GluA2 subunit (Boudreau, Reimers, Milovanovic, & Wolf, 2007; Conrad et al., 2008; Kourrich, Rothwell, Klug, & Thomas, 2007). GluA2-containing AMPARs are primarily Calcium Impermeable (so called CI-AMPARs). On the other hand, AMPARs without GluA2 subunits (GluA2-lacking AMPARs) have a higher affinity for glutamate and greater Calcium Permeability (so called CP-AMPARs). Because of these unique characteristics, CP-AMPARs also contribute to the induction of synaptic plasticity under some circumstances.

1.4.3 Effects of cocaine exposure on excitatory synapses

It has been hypothesized that drugs of abuse “hijack” the common synaptic plasticity mechanisms to induce adaptive neuronal changes, and some drug-induced neural adaptations underlie the key pathophysiologies of drug addiction. A number of cocaine-induced neural adaptations have been identified, which may be behaviorally important.

The alteration in synaptic transmission to the VTA may occur after a single exposure to cocaine. The AMPAR/NMDAR ratio in VTA DAergic neurons is increased after 1 day from a single exposure to cocaine. Following investigations reveal that up-regulation of AMPAR mediates this cocaine-induced alteration, and it may share the mechanism with NMDAR-dependent LTP observed in the VTA (Kauer & Malenka, 2007; Ungless, Whistler, Malenka, & Bonci, 2001; van Huijstee & Mansvelder, 2015). Also, this enhanced AMPAR transmission is mediated by insertion of GluA2-lacking AMPARs (Argilli, Sibley, Malenka, England, & Bonci, 2008; Bellone & Lüscher, 2006).

The NAc, a VTA projection region, also exhibits cocaine-induced cellular adaptations at excitatory synapses, although a single exposure to cocaine seems not to be sufficient to induce synaptic plasticity (Kourrich et al., 2007). For example, repeated exposure to cocaine followed by withdrawal causes the decreased basal extracellular glutamate levels in the NAc (Baker et al., 2003; Baker, Shen, & Kalivas, 2002; Pierce, Bell, Duffy, & Kalivas, 1996) and the decreased intrinsic excitability of NAcSh MSNs (Dong et al., 2006; Ishikawa et al., 2009; Kourrich & Thomas, 2009; Mu et al., 2010). On the other hand, other studies have shown an up-regulation of postsynaptic responsiveness in NAc excitatory synapses after long-term withdrawal from cocaine exposure (Boudreau et al., 2007; Boudreau & Wolf, 2005; Conrad et al., 2008; Kourrich et al.,

2007). It has been known that these synaptic adaptations following cocaine exposure are dynamic, depending on the drug regimens and withdrawal time. For example, non-contingent exposure to cocaine results in up-regulation of CI-AMPARs, starting from about 1 week after drug withdrawal. Contingent exposure to cocaine also up-regulates the synaptic CI-AMPAR levels after long-term withdrawal (not after short-term withdrawal), but the extended access to cocaine followed by a prolonged withdrawal period results in the increase of CP-AMPARs in the NAc (Wolf & Tseng, 2012). In addition, it has been reported that the increased glutamate transmission in the NAc is required for the reinstatement of cocaine seeking after withdrawal (Cornish, Duffy, & Kalivas, 1999; Cornish & Kalivas, 2000; Pierce & Wolf, 2013; Ping, Xi, Prasad, Wang, & Kruzich, 2008) (Additional discussion in Chapter 5).

It is difficult to compare the basal strength of excitatory synapses between different neurons, so changes in the AMPAR/NMDAR ratio are often used to detect alterations in excitatory synaptic strength. This normalization procedure is independent of the number of activated synapses, allowing relatively reliable estimation of the synaptic strength. Previous reports show that the AMPAR/NMDAR ratio decreases after short-term withdrawal from repeated non-contingent cocaine exposure or after a single challenge cocaine injection following about 2 weeks withdrawal period (Kourrich et al., 2007; Thomas, Beurrier, Bonci, & Malenka, 2001). After short-term withdrawal, the cell surface AMPARs stay at the basal level (Boudreau et al., 2007; Boudreau & Wolf, 2005). Meanwhile, it is observed that new NMDARs are inserted into the postsynaptic membrane of NAcSh MSNs after short-term withdrawal from repeated non-contingent cocaine exposure, and this alteration may contribute to the decreased AMPAR/NMDAR ratio (Y. H. Huang et al., 2009). On the other hand, after longer-term withdrawal from non-contingent exposure to cocaine, the AMPAR/NMDAR ratio increases in NAcSh MSNs (Kourrich et al., 2007), which

is accompanied by the up-regulation of AMPAR levels (Boudreau et al., 2007; Boudreau & Wolf, 2005).

1.5 INHIBITORY TRANSMISSION

Inhibitory synaptic transmission hyperpolarizes postsynaptic cells, thus functionally opposing excitatory synaptic transmission. The balance between EPSPs and inhibitory postsynaptic potentials (IPSPs), which are produced by excitatory synapses and inhibitory synapses respectively, is physiologically important. IPSPs can be temporary summed with EPSPs to comprise the depolarizing effect of EPSPs, affecting action potential firing. The refined balance between EPSCs/IPSCs allows neurons to maintain stable functional output. There are two types of inhibitory neurotransmitters in the CNS; glycine and γ -aminobutyric acid (GABA). Glycine is most common in the spinal cord, brain stem, and retina, while GABA is common in the adult mammalian brain. GABA receptors are classified in two types; ionotropic receptors and metabotropic receptors. Our present study mainly focuses on ionotropic receptors (i.e. GABA_A receptors), which mediate fast inhibitory synaptic transmission in the NAc.

1.5.1 GABAR

GABA is an amino acid that functions mainly as a neurotransmitter in inhibitory synaptic transmission. GABA does not penetrate through the blood-brain barrier, so it is synthesized in the brain. GABA is synthesized from glutamate, via L-glutamic acid decarboxylase (i.e. GAD) and pyridoxal phosphate (Petroff, 2002; Schousboe & Waagepetersen, 2007). Since the direction of

the flow of negatively charged chloride ions is most often into the cell, the membrane potential is hyperpolarized upon activation of GABARs. GABARs are pentamers, made of isoforms of three main subunits, α , β , and γ . Interestingly, GABAR α subunit shows a developmental shift, like NMDARs. Specifically, $\alpha 2/3$ subunits are predominant in young age and they are gradually replaced by $\alpha 1$ subunit as animals grow (Fritschy, Paysan, Enna, & Mohler, 1994; Wisden, Laurie, Monyer, & Seeburg, 1992)

1.5.2 The Excitatory Effect of GABA transmission

While GABA-mediated synaptic transmission is inhibitory in the adult brain, it is excitatory during early developmental stages. Most studies have been done in the neocortex and the hippocampus of the mammalian brain showing that the GABA's excitatory effect is common before the maturation of glutamatergic synapses (Ben-Ari, Gaiarsa, Tyzio, & Khazipov, 2007; Li & Xu, 2008). In young animals, the reversal potential of the chloride conductance is relatively depolarized because the intracellular concentration of chloride is high, making chloride ions exit the cell and leading to depolarization/excitation of the neuron when GABA_ARs are activated. In adult animals, the reversal potential is hyperpolarized below the resting membrane potential, and GABA_AR-mediated transmission becomes inhibitory because chloride ions enter the cell upon GABAR activation (Ben-Ari, 2002). Early expression of the sodium-potassium-2 chloride co-transporter (NKCC1), which transports sodium, potassium and chloride ions into the cell, and late expression of potassium-chloride- co-transporter (KCC2), which transports potassium and chloride ions to outside of the cell, together determine the developmental changes of intracellular chloride concentrations. NKCC1 is predominantly expressed in neurons of young animals and it makes the intracellular chloride concentration higher. On the other hand, expression of KCC2 is

dominant when animals get older and the intracellular chloride concentration becomes lower (Ben-Ari, 2002; Yamada et al., 2004). These two chloride transporters dynamically regulate the intracellular chloride concentration to shift the action of GABA from excitatory to inhibitory (Fukuda et al., 1998).

1.5.3 VTA GABAergic neurons

As described above, the VTA contains a large GABAergic cell population and it has been known that inhibitory synapses in the VTA have a critical role in controlling the firing rate of DAergic neurons. Also, Liu and the colleagues showed that LTP induction is facilitated in VTA DAergic neurons after repeated cocaine exposure and that this facilitation is due to the reduction of GABAR-mediated inhibition. Also, they found that enhanced GABA-mediated inhibition prevents the induction of LTP (Liu et al., 2005). Thus, GABAergic synaptic transmission can be targeted by drugs of abuse to produce addiction-related cellular and behavioral alterations.

After the invention of optogenetics, the Channelrhodopsin 2 (ChR2) can be selectively expressed in GABAergic neurons using Cre mice (Gad-Cre or VGAT-Cre), allowing researchers to selectively manipulate the VTA GABAergic neurons *in vivo* or *in vitro*. A study shows that optical activation of GABAergic neurons in the VTA reduces the activity of DAergic neurons *in vivo* (Tan et al., 2012). The same group has also applied this approach *in vivo* in a dynamic conditioned place aversion test. Specifically, Gad-Cre mice with intra-VTA floxed-ChR2 expression were optogenetically stimulated when they were exploring one of the two compartments and the stimulation was turned off as soon as the mice exited the compartment. As a result, the mice developed an aversion for the blue-light paired chamber. This result indicates a strong aversive learning effect resulting from the inhibition of DAergic neurons by activation of

local GABAergic neurons in the VTA (Creed, Ntamati, & Tan, 2014; Tan et al., 2012). In addition, another group used halorhodopsin to inhibit VTA GABAergic neurons, which increases activity of DAergic neurons (Bocklisch et al., 2013). These studies indicate that the local VTA GABAergic neurons have direct inhibitory connection to DAergic neurons to regulate reward-associated behaviors.

In addition to the local control, ~25% of the VTA GABAergic neurons also project outside of the VTA, including the NAc (Margolis, Lock, Hjelmstad, & Fields, 2006; Margolis, Lock, Chefer, et al., 2006). Also, the long-range GABAergic projection neurons are activated with the parallel DAergic projection from the VTA to the NAc (Carr & Sesack, 2000; Steffensen et al., 1998). Although, the role of this type of inhibitory neurons has not been investigated well, Brown et al. published a study focusing on this long-range GABA projection. Using Gad-Cre mice with intra-VTA floxed-ChR2 injection, this group showed that the long-range GABA projection from the VTA connect selectively to cholinergic interneurons (CINs) in the NAc. In addition to neuroanatomical evidence, their electrophysiological recording showed that inhibitory current was observed selectively in CINs, not MSNs, after activating VTA GABA axons in the NAc. Their subsequent *in vivo* recordings showed that activating VTA GABAergic neurons inhibits CINs activity. Moreover, they optogenetically stimulated VTA GABAergic neurons to pair the tone when mice received tone paired foot-shocks. This manipulation increased ability of the Gad-Cre mice to discriminate this conditioned tone from an unconditioned tone. Based on these observations, the authors concluded that VTA GABAergic projection only terminate on NAc CINs and this projection is implicated in associative learning (M. T. Brown et al., 2012).

Opposite to the results of this study, another study suggests that the long-range GABA projection neurons have synaptic connections with NAc MSNs. A study published in the same

year investigated the long-range GABA-mediated IPSCs using VGAT-Cre mice and recorded IPSCs in “NAc neurons” (van Zessen, Phillips, Budygin, & Stuber, 2012). They randomly chose NAc neurons to record without specifying their cell types, but these “NAc neurons” should be MSNs. In the NAc, only 1-2% of neurons are CINs, whereas >90% are MSNs. Whether VTA GABAergic projections innervate NAc MSNs? Solving this controversial yet important question is one of our research objectives (Chapters 2-5).

1.5.4 Inhibitory Synaptic Plasticity

Similar to excitatory synapses, GABAergic synapses are plastic, and undergo experience-dependent long-term bi-directional changes (Woodin & Maffei, 2011). Several forms of plasticity at inhibitory synapses have been reported, expressed either pre- or postsynaptically (Castillo, Chiu, & Carroll, 2011). A widely observed form of GABAergic plasticity involves alterations in the presynaptic GABA release, which, in many occasions, is triggered heterosynaptically. Heterosynaptic induction of GABAergic plasticity needs non-GABAergic participation and it is often from the nearby synapses. Similar to the plasticity at the excitatory synapses, available evidence indicates that endocannabinoid (eCB) and NMDAR play important roles for GABAergic plasticity. In addition, BDNF-mediated inhibitory LTP (iLTP) and NO-mediated iLTP are also reported in many brain regions. These forms of inhibitory plasticity are accompanied by postsynaptic alterations, including alterations in chloride transporter activity, receptor phosphorylation, or receptor trafficking (Castillo et al., 2011).

Inhibitory synapses play an important role in regulating neuronal excitation. The fine balance between excitatory and inhibitory synaptic inputs is critical for a stable functional state of a neuron, particularly for NAc MSNs, which do not have internal pace-making mechanisms.

Experience-dependent plasticity at GABAergic synapses may reshape the balance between excitatory and inhibitory synaptic inputs, altering the overall functional output of NAc MSNs and related circuits.

1.5.5 Effect of cocaine exposure on inhibitory synapses

At postsynaptic terminals of inhibitory GABAergic synapses, alterations of subunit composition, especially α subunits of GABA_ARs, are a common form of cellular adaptation. Recent evidence suggests that $\alpha 2$ -containing GABA_AR is essential for the induction of behavioral sensitization to cocaine (Dixon et al., 2010), whereas other results show that repeated non-contingent cocaine exposure increases the mRNA levels of several GABA_AR subunits and the protein level of $\alpha 1$ subunit (Kennedy et al., 2013). In addition, following non-contingent exposure to cocaine, the whole brain level of $\alpha 1$ subunits is reduced (Suzuki et al., 2000), which can be replaced by $\alpha 2/3$ subunits. The potential switch of $\alpha 1$ by $\alpha 2/3$ subunits in GABA_ARs prolongs the decay kinetics of IPSCs (Gingrich, Roberts, & Kass, 1995; Lavoie & Twyman, 1996; Verdoorn, 1994). Those results suggest that dynamic neural adaptations at inhibitory synapses may also occur after cocaine exposure, depending on the drug regimens (e.g., passive vs. active exposure) or withdrawal times.

GABA_AR trafficking has unique features. Only co-expression of α and β subunits, not the α/γ or β/γ subunit combination, in heterologous cells forms functional receptors and are expressed on the cell surface (Brickley, Cull-candy, & Farrant, 1999; Mortensen & Smart, 2006). Also, $\alpha 1$ deletion in mice causes compensatory up-regulation of receptors containing other α subunits (Kralic, Korpi, O' Buckley, Homanics, & Morrow, 2002; Kralic et al., 2006; Kralic, O' Buckley, et al., 2002; Sur et al., 2001). A study shows that the surface expression of GABA_AR $\alpha 1$ subunits

is decreased following repeated non-contingent exposure to cocaine (C.-C. Huang, Lin, & Hsu, 2007). Therefore, the altered postsynaptic GABA responsiveness can be mediated by altered surface expression of GABA_ARs or changes in subunit expression.

1.6 INTRINSIC PLASTICITY

The functional output of NAc MSNs is driven by integrated excitatory and inhibitory synaptic inputs and the output is gated by the intrinsic membrane excitability. The intrinsic membrane excitability determines whether and how many action potentials to fire upon membrane depolarization (Groenewegen, Wright, Beijer, & Voorn, 1999; O'Donnell, Greene, Pabello, Lewis, & Grace, 1999; Wilson & Groves, 1981).

Cocaine-induced alterations in membrane excitability have also been reported. The membrane excitability of NAcSh MSNs is decreased after short-term withdrawal from cocaine exposure (Dong et al., 2006). After long-term withdrawal from cocaine, the intrinsic membrane excitability of NAcSh MSNs remains low. At this withdrawal point, the surface levels of AMPARs are increased. The changes in excitatory synaptic transmission and the membrane excitability appear to cancel out each functionally. In addition, a previous study from our laboratory demonstrated that the membrane excitability of NAcSh neurons is regulated differently by different cocaine regimens. After short-term withdrawal from repeated i.p. cocaine injections or cocaine self-administration, the membrane excitability of MSNs is decreased. After long-term withdrawal from repeated non-contingent treatment of cocaine, the membrane excitability stays at low levels. On the other hand, the membrane excitability is back to the normal level in rats treated with the self-administration regimen. Upon re-exposure to cocaine after long-term withdrawal, the

membrane excitability is back to the normal level in rats treated with i.p. injections of cocaine. However, in rats treated with cocaine self-administration, the membrane excitability increased beyond the normal level upon cocaine re-exposure. These results suggest that the membrane excitability of NAc MSNs dynamically changes during different phases of cocaine experience (Mu et al., 2010).

1.7 FAST SYNAPTIC TRANSMISSIONS FROM THE VTA TO THE NAC

As described above, the VTA-to-NAc projection plays critical role in motivational responses. The VTA projections are mainly from DAergic/glutamatergic neurons, GABAergic neurons, and possibly also a small percentage of purely glutamatergic neurons (Carr & Sesack, 2000; Chuhma et al., 2004; Hnasko et al., 2010; Joyce & Rayport, 2000; Lavin et al., 2005; Nagai, McGeer, & McGeer, 1983; Nair-Roberts et al., 2008; Steffensen et al., 1998; Stuber et al., 2010; Sulzer et al., 1998; Tecuapetla et al., 2010). Both DAergic and GABAergic VTA neurons are activated simultaneously upon the emotional/motivational stimuli (Carr & Sesack, 2000; Steffensen et al., 1998). Whereas VTA-to-NAc DA transmission may play a central role in regulating the function of NAc neurons, fast glutamate and GABA transmissions provide direct and fast activation or inhibition of NAc neurons. Compared to DAergic transmission, glutamatergic and GABAergic transmissions within the VTA-to-NAc projections remain poorly understood. Especially for VTA-to-NAc GABAergic projections, results are inconsistent (M. T. Brown et al., 2012; van Zessen et al., 2012). Because of the lack of basic characterization, little is known about how these fast VTA-to-NAc transmissions regulate the functional output of NAc MSNs, and whether exposure to drugs of abuse reshapes these fast transmissions.

In following two chapters (Chapter 2 and 3), we will characterize VTA-to-NAc GABAergic transmission, the regulation of the GABAergic transmission by co-released DA, and the adaptive changes of this transmission by cocaine experience. In parallel, we will examine the glutamatergic transmission because it will provide a direct comparison between GABAergic and glutamatergic transmissions for several key aspects of the VTA-to-NAc transmissions. In Chapter 4, we will examine the overall inhibitory effect of cocaine experience to NAc MSNs by focusing on the characterization of inhibitory synaptic transmission, the balance of excitatory and inhibitory synaptic inputs, and the overall output of NAc MSNs after cocaine exposure.

2.0 FAST SYNAPTIC TRANSMISSION FROM THE VENTRAL TEGMENTAL AREA TO THE NUCLEUS ACCUMBENS AND THE EFFECT OF COCAINE EXPOSURE¹

2.1 INTRODUCTION

Emotional and motivational responses are critically regulated by the brain reward system, which comprises reciprocal projections among the VTA, NAc and other related brain regions (Phillips, 1984; Wise, 1987). Pathophysiological alterations of these projections may critically contribute to a variety of emotional and motivational disorders, such as drug addiction and depression (Hyman et al., 2006; Wise, 1996). Within the complex neural network of the brain reward system, the synaptic projection from VTA DAergic neurons to NAc MSNs is relatively well defined for its role in the acute rewarding effect and emotion/motivation-associated learning (Hyman et al., 2006; Kelley, 2004). However, DAergic neurons and possibly other neuronal types within the VTA also project excitatory glutamatergic axons onto NAc MSNs (Chuhma et al., 2004; Hnasko et al., 2010; Joyce & Rayport, 2000; Lavin et al., 2005; Nair-Roberts et al., 2008; Stuber et al., 2010; Sulzer et al., 1998; Tecuapetla et al., 2010). Furthermore, GABAergic neurons in the VTA, which are intermixed with DAergic neurons, receive synaptic inputs from the same brain regions, and project to NAc MSNs in parallel with DAergic neurons (Carr & Sesack, 2000; Nagai et al., 1983; Steffensen et al., 1998). Compared with the modulatory role of DA, fast excitatory

¹ The work presented in Chapter 2 is from preliminary data by M. Otaka and from “Exposure to cocaine regulates inhibitory synaptic transmission from the ventral tegmental area to the nucleus accumbens,” M. Ishikawa, M. Otaka, P.A. Neumann, Z. Wang, J.M. Cook, O.M. Schluter, Y. Dong, and Y.H. Huang, 2013, *J Physiol*, 591(Pt 19), 4827-4841.

and inhibitory VTA-to-NAc synaptic transmissions may exert more direct and timing-specific control over NAc MSNs and their functional output. However, the cellular properties of these fast VTA-to-NAc synaptic transmissions and their cellular responses to drugs of abuse have not been well characterized.

The NAc contains two subregions, the shell and core, receiving projections from the VTA with a medial-to-lateral topographical pattern. Specifically, DAergic neurons that project to the ventromedial NAcSh are primarily located in the posteromedial VTA, including the paranigral nucleus (PN), the central linear nucleus and the medial part of the parabrachial pigmented area (PBP), whereas the DAergic cell bodies projecting to the NAcCo are primarily located in the anterolateral part of the VTA, in particular the lateral part of the PBP (Ikemoto, 2007). However, from the medial to lateral VTA–nigra complex, the co-expression of VGLUT2 with TH gradually decreases (Kawano et al., 2006). These topographical features suggest that the NAcSh receives more VTA glutamate/DA co-release than the NAcCo. The differences in these innervation patterns probably contribute to the differential roles of the NAcSh and NAcCo in cocaine-induced behaviors. One hypothesis is that the NAcSh is preferentially involved in the emotional and motivational aspects of the addictive state, whereas the NAcCo is preferentially involved in the habitual learning aspect (Kelley, 2004). Thus far, a number of previous studies have been devoted to the characterization of the VTA-to-NAcSh excitatory synapses (Y. H. Huang, Ishikawa, et al., 2011; Stuber et al., 2010), leaving the VTA-to-NAcCo excitatory synapses underexplored. Here, we examine and compare VTA glutamatergic projections to the NAcSh and NAcCo, with more detailed characterization on VTA-to-NAcCo synapses. Compared with glutamatergic projections, VTA-to-NAc GABAergic projections have been less well characterized. A recent study has reported that activation of VTA-to-NAc GABAergic projection fails to elicit responses in 96.6%

of NAc MSNs (without discriminating between NAcSh and NAcCo) (M. T. Brown et al., 2012). However, another study detected VTA-to-NAc GABAergic synaptic responses in the NAc (van Zessen et al., 2012). In this chapter, we focus on NAcCo MSNs to examine VTA-to-NAc GABAergic projection. Using viral-mediated in vivo expression of ChR2 combined with pharmacological and electrophysiological manipulations, we dissect and characterize the fast excitatory and inhibitory synaptic transmission from the VTA to the NAc.

2.2 MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats were purchased from the Simonsen (CA, USA) and Harlan Laboratories (MD, USA). In a set of experiments, the *Gad2*-IRES-Cre (*Gad2*^{tm2(cre)zjh}; Jackson, Laboratories; ME, USA) and TH-IRES-Cre (B6.129×1-Th^{tm1(cre)Te}/Kieg, The European Mouse Mutant Archive, Munich, Germany) (Lindeberg et al., 2004) mouse lines were used. All following procedures were performed by strictly following the standard procedures approved by the Institutional Animal Care and Use Committee at Washington State University, the University of Pittsburgh, and European Neuroscience Institute.

Viral vectors

ChR2 fused to Venus (ChR2Y; Addgene plasmid 20071) or mCherry (ChR2R) was expressed from an Adeno-associated viral vector (AAV) 2 with AAV2 internal repeats (Atasoy, Aponte, Su, & Sternson, 2008; Petreanu, Mao, Sternson, & Svoboda, 2009; Suska, Lee, Huang, Dong, & Schlüter, 2013). AAV2-flexed-ChR2R expression was induced specifically in Cre

recombinase-expressing neurons, where the inverted expressing cassette was flipped. In this study, AAV2-flexed-ChR2R was used in Gad-Cre or TH-Cre mouse lines, and AAV2-ChR2Y was used for rats. AAV2 stereotypic AAV vectors were generated in HEK293T cells and purified by discontinuous iodixanol gradient centrifugation.

Surgery

Intra-VTA or intra-NAcSh viral injections were performed when animals were at a postnatal age of 26–34 days. For in vivo delivery of viral vectors, animals were anaesthetized with a mixture of ketamine/xylazine (100/10 mg/kg) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). For the experiments using rats, a 28-gauge injection needle was used to bilaterally inject 1 μ l (0.2 μ l/min) of the AAV-ChR2Y solution via a Hamilton syringe into the VTA (AP -5.00 , ML ± 0.90 , DV -7.65) or the NAcSh (AP $+1.55$, ML ± 0.80 , DV -6.50) using a Thermo Orion M365 pump (Thermo Scientific, Barrington, IL, USA). Injection needles were left in place for 5 min following injections. For mice, a 28-gauge needle was used to bilaterally inject 1 μ l (0.2 μ l/min) of the virus solution into the VTA (AP -3.44 , ML ± 0.48 , DV -4.4).

Cocaine Administration

After the recovery period (about 2 weeks) from the surgery, animals received repeated i.p. injections of cocaine (15 mg/kg in saline for 5 consecutive days) or saline in a novel environment, as described previously (T. E. Brown et al., 2011; Ishikawa et al., 2009; Mu et al., 2010). Rats were placed back to the home cage for withdrawal. This total timeline allowed about 3 weeks to pass following the viral injection, allowing for adequate viral-mediated protein expression.

NAc slice preparation and electrophysiology

The animal was decapitated following deep isoflurane anaesthesia. The brain was removed and glued to a block and sliced with a vibratome in 4 °C cutting solution containing (in mM) N-methyl-D-glucamine (135), KCl (1), KH₂PO₄ (1.2), CaCl₂ (0.5), MgCl₂ (1.5), choline-HCO₃ (20) and glucose (10), saturated with 95% O₂/5% CO₂, with the pH adjusted to 7.4 with HCl. The brain slices were incubated in artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl (119), KCl (2.5), CaCl₂ (2.5), MgCl₂ (1.3), NaH₂PO₄ (1), NaHCO₃ (26.2) and glucose (11), saturated with 95% O₂/5% CO₂ at 37 °C for 30 min and then allowed to recover for at least 30 min at room temperature before electrophysiological recordings. Signature anatomical landmarks (e.g. the anterior commissure and lateral ventricle) were used to delineate the NAcSh and NAcCo. 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, Hamden, CT, USA) before entering the recording chamber. Recordings were made under visual guidance (40x, differential interference contrast optics) with electrodes (3–5MΩ) filled with cesium based internal solution containing (in mM) CsCH₃O₃S (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.

To record EPSCs, picrotoxin (PTX; 100 μM) was included in the external perfusion aCSF to block GABA_AR-mediated currents. To evoke VTA-to-NAc synaptic transmission, axons expressing ChR2 were stimulated by 473 nm DPSS laser (IkeCool, Los Angeles, CA, USA) coupled to a 62.5 μm optic fiber. An optical stimulation of <1 msec duration was used to stimulate the ChR2-expressing axons. EPSCs were recorded at –70 mV in the presence of PTX. To measure AMPAR/NMDAR ratio, the NMDAR-mediated components were measured at +50 mV and the AMPAR-mediated EPSCs were measured at –70 mV. Operationally, the amplitude of AMPAR

EPSCs was measured at the peak of EPSCs at -70 mV, and the amplitude of NMDAR EPSCs was measured at $+50$ mV, 30 ms after the onset of EPSCs; at this time point AMPAR EPSCs were largely inactivated (Fig. 2). This measurement is similar to some (Dong et al., 2004) but different from other (Kourrich et al., 2007; Mameli et al., 2009) previous studies measuring the AMPAR/NMDAR ratio. Specifically, the peak amplitudes of AMPAR-mediated EPSCs were larger at -70 mV than at $+40$ or $+50$ mV, resulting in larger absolute values of the AMPAR/NMDAR ratio. Because the AMPAR/NMDAR ratio is a relative measurement, the absolute values of this ratio do not carry physiological significance, whereas the changes in this ratio under the same recording conditions reflect synaptic alterations. IPSCs were recorded at $+10$ mV, which was around the reversal potential of EPSCs. To measure the decay kinetics of IPSCs, the time elapsed from the peak amplitude of IPSCs to one-third of the peak amplitude ($T_{1/3}$) was used. The value of $T_{1/3}$ is an operational estimate of the time constant (i.e. $T_{36.8\%}$) assuming that the decay of IPSCs follows single-exponential decay kinetics.

For all recordings, the series resistance was 8–14M Ω and was left uncompensated. The series resistance was monitored continuously during all recordings, and a change beyond 20% was not accepted for data analysis. Synaptic currents were recorded with a MultiClamp 700A amplifier (Molecular Devices), filtered at 3 kHz, amplified five times and then digitized at 20 kHz with a Digidata 1322A analogue-to-digital converter (Molecular Devices, CA, USA).

Drugs

Chemicals were purchased from Tocris Bioscience (Bristol, UK) and Sigma-Aldrich (St Louis, MO, USA) unless specified otherwise.

Data analysis and statistics

All results are shown as the mean \pm SEM. Statistical significance was assessed using one- or two-factor ANOVA with Bonferroni post-tests or two-tailed t test. One to four cells were recorded from each animal. The total number of cells is presented as 'n' and the total number of animals as 'm'. Thus, the sample size is presented as n/m. Cell-based statistics were performed and presented for all results.

2.3 RESULTS

To electrophysiologically dissect fast excitatory and inhibitory synaptic transmission from the VTA to the NAc, we injected AAV2-ChR2Y into the VTA of rats (Petreanu et al., 2009). To selectively target VTA DAergic or GABAergic neurons, we used the TH-Cre or GAD-Cre mice with intra-VTA injections of AAV2-flexed-ChR2R.

Three weeks following virus injection, animals were sacrificed to obtain horizontal brain slices. Within these slices, strong fluorescence signal was observed in the VTA, indicating the expression of ChR2 (Fig. 1A). In addition, a large number of ChR2-positive neural fibers were observed projecting from the VTA to the forebrain including the NAc (Fig. 1A). By laser-mediated activation of ChR2, the VTA-to-NAc synaptic transmission was selectively activated and examined.

Fast excitatory synaptic transmission

In wild-type rats and TH-cre mice with intra-VTA injection of ChR2-expressing viruses, fast postsynaptic currents were consistently elicited in NAcCo neurons by brief laser exposures

(<1 ms, $\lambda=473$ nm) directed towards the ChR2-expressing fibers (in the presence of 100 μ M PTX to inhibit GABA_AR-mediated response) (Fig. 1B). These synaptic currents exhibited a short delay from optical stimulation to the onset of the current (rats, 2.54 ± 0.12 ms; mice, 2.33 ± 0.08 ms; Fig. 1C), fast activation (time to peak current: rats, 6.17 ± 0.24 ms; mice, 5.77 ± 0.25 ms; Fig. 1D) and inactivation (time to decay to one-third of peak amplitude: rats, 7.03 ± 0.42 ms; mice, 6.10 ± 0.46 ms; Fig. 1E). These synaptic parameters are consistent with those of monosynaptic transmission evoked using optogenetic stimulation in the striatum (Tritsch et al., 2012). Furthermore, these postsynaptic currents could be blocked by the AMPAR-selective antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX; 5 μ M) in both rat (n=5; Fig. 1F) and TH-Cre mouse (n=5; Fig. 1G) preparations, indicating that they were AMPAR-mediated EPSCs.

In our experimental preparations, in addition to triggering glutamatergic transmission, optical activation of VTA-to-NAcCo projections was also likely to trigger DA release, which may, in turn, tonically regulate VTA-to-NAcCo synaptic transmission. However, co-perfusion of the DA D1 and D2 class receptor antagonists SCH23390 (1 μ M) and eticlopride (3 μ M) did not affect either the peak amplitude ($p=0.38$ before vs. during perfusion) or the paired-pulse ratio (PPR; the ratio of the peak amplitude of the second EPSC over the peak amplitude of the first EPSC) ($p=0.57$ before vs. during perfusion) of the evoked EPSCs at the stimulation frequency of 0.1Hz (Fig. 1I, J). Taken together, this set of results shows that, similar to the optogenetic activation of VTA-to-NAcCo DAergic projection in transgenic mice, activation of the VTA-to-NAcCo projection triggered monosynaptic EPSCs, which were not affected by concurrent DAergic signaling under our experimental conditions.

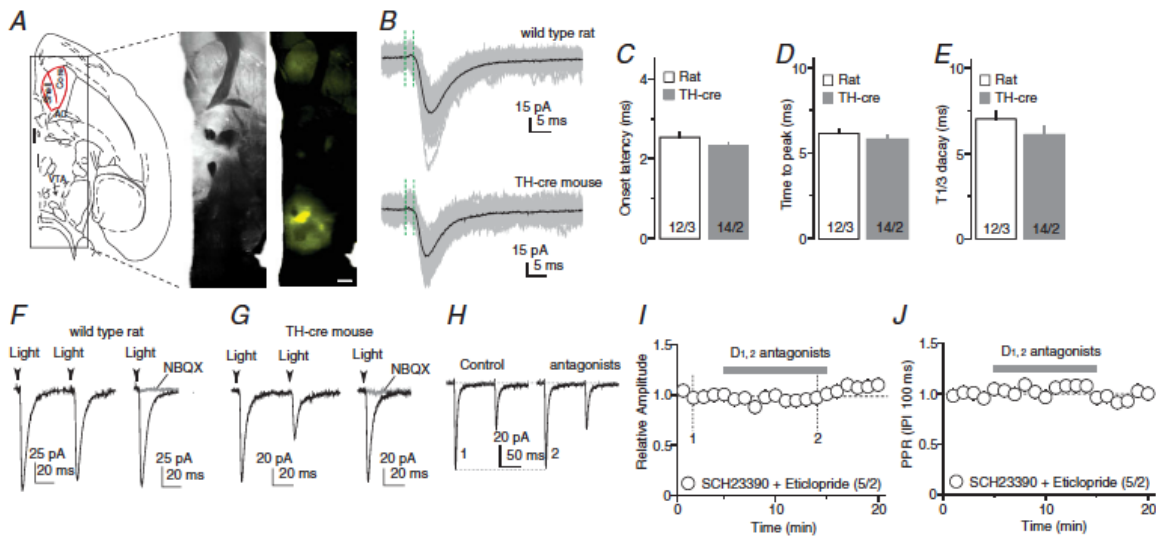


Figure 1. VTA-to-NAc Excitatory Synaptic Transmission.

A. Diagram (left) and images (right) showing that intra-VTA injection of ChR2-expressing AAV2 resulted in ChR2-YFP expression in the VTA and its projections. Images were taken from the differential interference contrast microscope used for electrophysiological recordings of this study.

B. Optogenetically evoked synaptic currents in example NAcCo neurons from a rat (top) with intra-VTA injection of AAV-ChR2Y and a TH-Cre mouse (bottom) with intra-VTA injection of AAV-flexed-ChR2R. Recordings were made by holding the membrane potential at -70 mV in the presence of PTX (100 μ M). The time elapsed from the artifacts by optogenetic stimulation (indicated by the first green vertical dashed line) to the initiation of the evoked synaptic currents (indicated by the second dashed line) was operationally used to measure the delay of synaptic transmission.

C. Summarized result showing that the delays of optogenetically evoked VTA-to-NAcCo synaptic transmission were short, consistent with monosynaptic transmission; they were not different between rats and TH-Cre mice.

D. Summarized results showing that the activation kinetics (operationally measured as the time elapsed from the initiation to the peak of evoked synaptic currents) of optically evoked synaptic currents were fast and not different between rats and TH-Cre mice with intra-VTA injections of AAV-flexed-ChR2R.

E. Summarized results showing that the decay kinetics (operationally measured as the decay time from the peak to one-third of the peak) of optically evoked synaptic currents were fast and were not different between rats and TH-Cre mice.

F. Examples showing that, in rats, optogenetic stimulations evoked synaptic currents from the VTA-to-NAcCo afferent (left), which were completely inhibited by the AMPAR-selective antagonist NBQX (right), indicating that they were EPSCs.

G. Examples showing that, in TH-cre mice, optogenetic stimulations evoked synaptic currents from the VTA-to-NAcCo afferent (left), which were completely inhibited by the AMPAR-

selective antagonist NBQX (right), indicating that they were EPSCs.

H. Example EPSCs from VTA-to-NAcCo synapses in a rat before and after perfusion of the dopamine D1 and D2 class receptor antagonists SCH23390 and eticlopride.

I. Summarized results showing that the inhibition of D1 and D2 receptors did not affect the EPSC amplitude from VTA-to-NAcCo synapses.

J. Summarized results showing that the inhibition of D1 and D2 receptors did not affect the PPR (IPI=100 ms) of EPSCs from VTA-to-NAcCo synapses.

After establishing the VTA-to-NAc excitatory transmission in slices, we examined whether it was affected after 1 day of withdrawal from repeated cocaine exposure. We first measured the AMPAR/NMDAR ratio as an indicator of potential postsynaptic alterations. Because AMPAR and NMDAR EPSCs were simultaneously elicited by the same presynaptic stimulation, changes in the AMPAR/NMDAR ratio should be largely attributable to altered postsynaptic receptors (i.e. AMPARs, NMDARs or both). In this experiment, both VTA-to-NAcCo and VTA-to-NAcSh excitatory synaptic transmissions were examined using the rat preparation. AMPAR-mediated EPSCs were measured at -70 mV and NMDAR-mediated components were measured at $+50$ mV. Cocaine treatment did not alter the AMPAR/NMDAR ratio at either the VTA-to-NAcCo or VTA-to-NAcSh synapse (Saline_NAcCo, 2.14 ± 0.25 , $n/m=8/5$; Saline_NAcSh, 1.41 ± 0.13 , $n/m=12/6$; Cocaine_NAcCo, 2.60 ± 0.43 , $n/m=8/5$; Cocaine_NAcSh, 1.94 ± 0.36 , $n/m=9/5$; $F=2.70$, $p=0.06$, one-way ANOVA; Fig. 2A, B). Notably, the AMPAR/NMDAR ratio appeared to be lower at VTA-to-NAcSh synapses relative to VTA-to-NAcCo synapses in both saline- and cocaine-exposed groups, but no statistical significance was detected. We next examined the potential presynaptic effects of cocaine exposure on VTA-to-NAcCo excitatory synapses by recording paired EPSCs (Interpulse interval: IPI=50 ms) (Fig. 2C, D). The PPR was similar at VTA-to-NAcCo excitatory synapses between saline and cocaine-treated rats (Saline, 0.60 ± 0.03 , $n/m=8/5$; Cocaine, 0.69 ± 0.06 , $n/m=13/5$; $p=0.8$, t test; Fig. 2C, D). It is possible that presynaptic alterations cannot be detected using PPR analysis at a single IPI. As such, we next examined the PPR at four different IPIs (25, 50, 100 and 200 ms), and our results again showed no significant difference at VTA-to-NAcCo excitatory synapses between saline- and cocaine-treated animals after 1 day of withdrawal ($F_{(1,116)}=0.005$, $p=0.944$, treatment effect; $F=0.314$, $p=0.815$, IPI \times treatment; two-factor ANOVA; Fig. 2E, F). However, a modest but significant decrease in the PPR was detected after

21 days of withdrawal ($F_{(1,158)}=6.77$, $p=0.01$, treatment effect; $F=0.146$, $p=0.932$, IPI \times treatment; two-factor ANOVA; Fig. 2G, H). Thus, similar to the prefrontal cortex-to-NAc afferent (Suska et al., 2013), exposure to cocaine may induce an increase in the presynaptic release of neurotransmitters at VTA-to-NAcCo synapses. It is worth noting that, in our experimental conditions, the PPR returned to the basal level (i.e. 1) after ~ 1 s, are recovery slower than that analyzed using electrical stimulation (Kourrich et al., 2007).

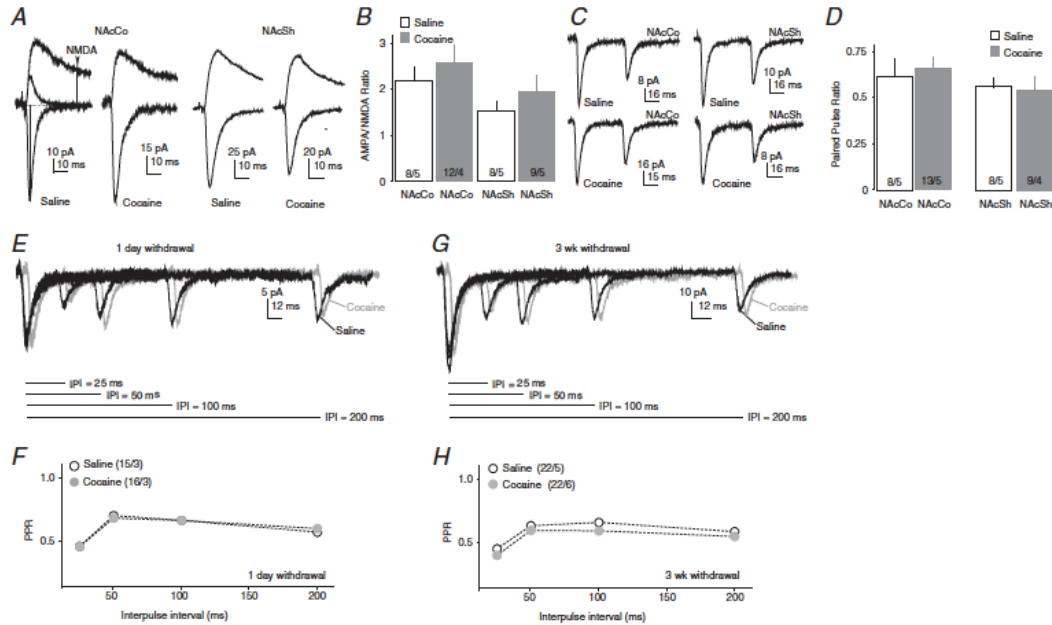


Figure 2. VTA-to-NAc fast excitatory synaptic transmission following i.p. cocaine administration.

A. Example EPSCs recorded at -70 and $+50$ mV from VTA-to-NAcCo (left) and VTA-to-NAcSh (right) synapses from saline- and cocaine-treated rats. In some recordings, AMPAR-mediated EPSCs were pharmacologically isolated at $+50$ mV (left), which decayed rapidly to the baseline. Operationally, the amplitude of AMPAR EPSCs was measured at the peak of EPSCs at -70 mV, and the amplitude of NMDAR EPSCs was measured at $+50$ mV at 30 ms after the onset of EPSCs, at which time point AMPAR EPSCs had largely returned to baseline.

B. Summarized results showing that, after 1 day of withdrawal from repeated i.p. injections of cocaine, the AMPAR/NMDAR ratio was not altered within the VTA-to-NAcCo/Sh projection.

C. Example EPSCs elicited by paired-pulse optical stimulations in NAcCo (left) and NAcSh (right) MSNs from saline-treated (top) and cocaine-treated (bottom) rats.

D. Summarized results showing that the PPR (IPI=50 ms) at VTA-to-NAcCo or VTA-to-NAcSh excitatory synapses was not altered 1 day after repeated exposure to cocaine (15 mg/kg per day for 5 consecutive days, i.p. injection).

E. Example EPSCs elicited from rat VTA-to-NAcCo synapses after 1 day of withdrawal by paired-pulse optical stimulations with IPI of 25, 50, 100 or 200 ms.

F. Summarized results showing that the PPRs of EPSCs from VTA-to-NAcCo synapses were not affected after 1 day of withdrawal from cocaine exposure.

G. Example EPSCs elicited from rat VTA-to-NAcCo synapses after 21 days of withdrawal by paired-pulse optical stimulations with IPI of 25, 50, 100 or 200 ms.

H. Summarized results showing that the PPRs of EPSCs from VTA-to-NAcCo synapses were modestly but significantly enhanced after 21 days of withdrawal from cocaine exposure. n/m, number of cells/number of animals.

In addition to the VTA-to-NAc projection, the NAc receives extensive excitatory projections from a large number of limbic and paralimbic brain regions, including the PFC, amygdala, thalamus and hypothalamus (Phillipson & Griffiths, 1985; Sesack & Grace, 2010). By random sampling of NAc excitatory synapses, extensive previous studies have demonstrated dynamic cocaine-induced alterations in the overall excitatory transmission onto NAc MSNs. The lack of cocaine-induced effects on VTA-to-NAc excitatory synapses led us to speculate that VTA-to-NAc excitatory synapses respond differently to cocaine treatment. To test this speculation, we sequentially recorded light-evoked VTA-to-NAc excitatory transmission and electrically evoked, randomly sampled excitatory transmission from the same NAcCo neurons. We found that light-evoked EPSCs readily reached a plateau amplitude as the stimulation intensity was increased (46.0 ± 6.2 pA, $n=37$). In addition, light-evoked EPSCs at VTA-to-NAcSh synapses were larger than those at VTA-to-NAcCo synapses (182.0 ± 35.7 pA, $n=7$; $p < 0.001$, t test). However, the peak amplitude of electrically induced (randomly sampled) excitatory monosynaptic transmission exhibited a wide range and could be as large as several thousands of pA upon strong stimulations (Fig. 3A). Furthermore, the postsynaptic properties, measured by the AMPAR/NMDAR ratio, were also different between VTA-to-NAc and randomly sampled excitatory synapses. Specifically, the AMPAR/NMDAR ratio was significantly higher at randomly sampled excitatory synapses than at VTA-to-NAcCo synapses in both saline-exposed (AMPA/NMDAR ratio from electrical stimulation relative to optical stimulation, 2.40 ± 0.25 ; $p < 0.01$, $n/m=8/5$; paired t test) and cocaine-exposed (1 day of withdrawal) (AMPA/NMDAR ratio from electrical stimulation relative to optical stimulation, 1.92 ± 0.34 , $n/m=8/5$; $p < 0.05$, paired t test; Fig. 3B, C) rats. Further analysis revealed that this synapse type-based difference in the AMPAR/NMDAR ratio was not affected by exposure to cocaine ($p=0.25$, t test). These results suggest that VTA-to-NAc excitatory synaptic

transmission is not likely to be a major contributor to the overall excitatory inputs onto NAc MSNs, exhibits different compositions of postsynaptic receptor populations compared with other excitatory synapses, and is probably resistant to changes upon cocaine exposure.

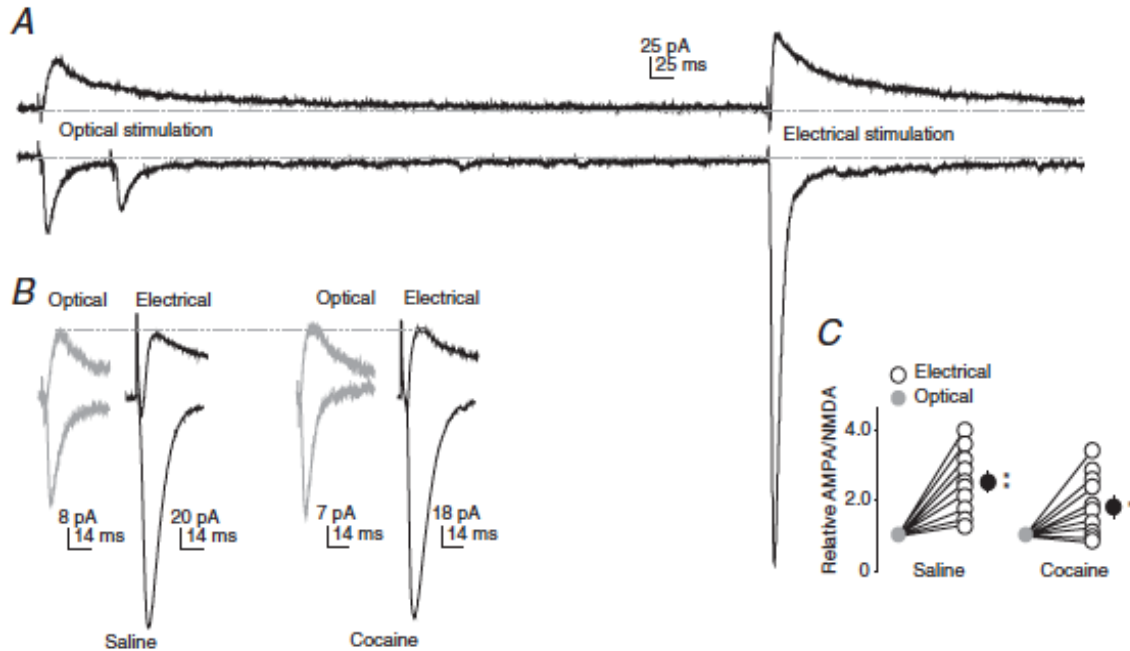


Figure 3. Different synaptic properties between VTA-to-NAcCo excitatory synapses and randomly sampled excitatory synapses on NAcCo MSNs.

A. Example AMPAR- and NMDAR-mediated EPSCs recorded at +40 mV (top) and -70 mV (bottom) from VTA-to-NAcCo synapses by optical stimulation (left) and randomly sampled synapses by electrical stimulation (right) from the same NAcCo neuron in a saline-exposed rat. Large EPSCs could be evoked from randomly sampled synapses upon strong stimulation, whereas EPSCs from VTA-to-NAcCo synapses were typically small and insensitive to the intensity of optical stimulation.

B. Examples of normalized EPSCs (to optically evoked NMDAR-mediated components) from VTA-to-NAcCo synapses and randomly sampled NAcCo synapses in saline- and cocaine-exposed rats.

C. Summarized results showing that the AMPAR/NMDAR ratio at VTA-to-NAcCo excitatory synapses is lower than that in randomly sampled excitatory NAcCo synapses, and this difference was not altered after 1 day of withdrawal from exposure to cocaine.

Fast inhibitory synaptic transmission

Compared with glutamatergic projections, VTA-to-NAc GABAergic projections have been characterized less consistently. A recent study has shown that the VTA-to-NAc GABAergic projection primarily synapses on CINs, and spares the majority of NAc MSNs (NAcSh or NAcCo, not specified) (M. T. Brown et al., 2012). However, another study detected VTA-to-NAc GABAergic synaptic responses in NAc neurons (van Zessen et al., 2012) and our preliminary and published data showed the consistent GABAergic responses in NAc MSNs (Figure 4A). Consistently, our subsequent work detects VTA-to-NAc GABAergic synaptic responses in the majority of NAcCo MSNs, (See Fig. 7 as additional evidence). Thus, our study focuses on NAcCo MSNs to examine the VTA-to-NAc GABAergic projection. Using similar approaches, we recorded optically evoked fast synaptic currents from VTA-to-NAcCo synapses at the holding potential of +10 mV, which was close to the reversal potential of AMPAR- and NMDAR-mediated EPSCs, thus minimizing the potential contamination from EPSCs. Some recordings were also made at +10 mV in the presence of NBQX, and the recorded IPSCs were no different in amplitude or kinetics from those recorded without the application of NBQX. Data were thus pooled for the following analysis. In wild-type rats and GAD-cre mice with intra-VTA injections of Chr2-expressing viruses, light-evoked IPSC-like currents were recorded from NAcCo MSNs (Fig. 4A, B). These synaptic currents exhibited short-delay onset (rats, 2.30 ± 0.16 ms; mice, 1.90 ± 0.14 ms; Fig. 4C), fast activation (time to peak: rats, 6.60 ± 0.44 ms; mice, 6.10 ± 0.41 ms; Fig. 2D) and inactivation (time to decay to one-third of peak amplitude: rats, 15.90 ± 1.64 ms; mice, 15.20 ± 0.67 ms; Fig. 4E). These synaptic parameters are consistent with those of monosynaptic transmission evoked using optogenetic stimulation in the striatum (Tritsch et al., 2012). Furthermore, these postsynaptic currents could be consistently evoked by optical stimulations and could be completely

inhibited by the GABA_AR-selective antagonist PTX (100 μ M) in both the rat (n=5; Fig. 4F) and GAD-Cre mouse (n=5; Fig. 4G) preparations, indicating that they were GABA_AR-mediated IPSCs. In addition, co-perfusion of the DA D1 and D2 class receptor antagonists SCH23390 (1 μ M) and eticlopride (3 μ M) did not affect either the peak amplitude (p=0.57 before vs. during perfusion) or the PPR (p=0.38 before vs. during perfusion) of evoked IPSCs, suggesting that VTA-to-NAcCo co-release of DA does not tonically regulate the GABAergic transmission under the current experimental conditions (i.e. at 0.1 Hz) (Fig. 4I, J). Taken together, this set of results shows that, similar to the optogenetic activation of VTA-to-NAcCo DAergic projection in transgenic mice, activation of the VTA-to-NAcCo projection triggers monosynaptic IPSCs, which were not affected by concurrent DAergic signaling under our experimental conditions.

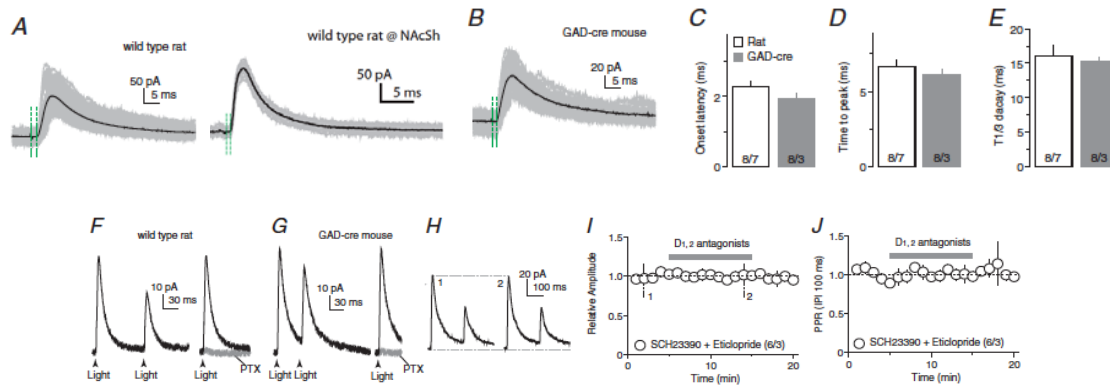


Figure 4. VTA-to-NAc Inhibitory Synaptic Transmission.

A and B. Optogenetically evoked synaptic currents in example NAc neurons from a rat with intra-VTA injection of AAV2-ChR2Y (**A**) and a GAD-Cre mouse with intra-VTA injection of AAV2-flexed-ChR2R (**B**). Recordings were made by holding the membrane potential at +10 mV, which was close to the reversal potential of EPSCs, resulting in minimal EPSCs. The time elapsed from the artifacts by optogenetic stimulation (indicated by the first green vertical dashed line) to the initiation of the evoked synaptic currents (indicated by the second dashed line) was operationally used to measure the delay of synaptic transmission.

C. Summarized result showing that the delay of optogenetically evoked VTA-to-NAcCo synaptic transmission was short, which was consistent with monosynaptic transmission; the onset latency was not different between wild-type rats and GAD-Cre mice.

D. Summarized results showing that the activation kinetics (operationally measured as the time elapsed from the initiation to the peak of evoked synaptic currents) of optically evoked synaptic currents were fast and not different between rats and GAD-Cre mice.

E. Summarized results showing that the decay kinetics (peak to one-third peak decay time) of optically evoked synaptic currents were fast and not different between rats and GAD-Cre mice.

F. Examples showing that, in the rat, optogenetic stimulations evoked synaptic currents from the VTA-to-NAcCo afferent (left), which were completely inhibited by the GABA_A-selective antagonist PTX (right), indicating that they were GABA_A-mediated IPSCs.

G. Examples showing that, in the GAD-Cre mouse, optogenetic stimulations evoked synaptic currents from the VTA-to-NAcCo afferent (left), which were completely inhibited by the GABA_A receptor-selective antagonist PTX (right), indicating that they were GABA_A-mediated IPSCs.

H. Example EPSCs from VTA-to-NAcCo synapses in a rat before and after application of the dopamine D1 and D2 class receptor antagonists SCH23390 (1 μ M) and eticlopride (3 μ M).

I. Summarized results showing that the inhibition of D1 and D2 receptors did not affect the IPSC amplitude from VTA-to-NAcCo synapses.

J. Summarized results showing that the inhibition of D1 and D2 receptors did not affect the PPR (IPI=100 ms) of IPSCs from VTA-to-NAcCo synapses.

After verifying the above experimental approach, we examined whether exposure to cocaine altered VTA-to-NAcCo inhibitory synaptic transmission. On withdrawal day 1 from repeated exposure to cocaine, a significant increase in the PPR was observed at VTA-to-NAcCo synapses ($F_{(1,144)}=34.57$, $p<0.01$, treatment effect; $F=0.19$, $p=0.90$, IPI \times treatment; two-factor ANOVA; Fig. 5A, B), suggesting an inhibition of presynaptic release. In parallel, we also examined the inhibitory projection from the NAcSh to NAcCo, which provides important lateral inhibitory control of NAcCo neurons (Morgan, Soto, Wong, & Kerschensteiner, 2011). This was achieved by intra-NAcSh injection of AAV-ChR2Y (Fig. 5C). In saline-treated animals, NAcSh-to-NAcCo inhibitory synapses exhibited a significantly higher PPR relative to VTA-to-NAcCo inhibitory synapses at all four tested IPIs (25, 50, 100 and 200 ms) ($F_{(1,100)}=194.4$, $p<0.001$, treatment effect; $F=0.72$, $p=0.54$, IPI \times treatment; two-factor ANOVA), suggesting a higher presynaptic release probability at VTA-to-NAcCo inhibitory synapses. Furthermore, exposure to cocaine (examined on withdrawal day 1) did not affect the PPR at NAcSh-to-NAcCo synapses ($F_{(1,156)}=0.08$, $p=0.77$, treatment effect; $F=0.09$, $p=0.97$, IPI \times treatment; two-factor ANOVA; Fig. 5D, E). However, the effect of cocaine on the PPR of VTA-to-NAcCo inhibitory synapses was no longer detectable after 21 days of withdrawal, suggesting that this effect was relatively transient ($F_{(1,136)}=2.08$, $p=0.152$, treatment effect; $F=0.34$, $p=0.80$, IPI \times treatment; two-factor ANOVA; Fig. 5F, G). Thus, presynaptic release of VTA to NAcCo GABAergic transmission was probably targeted by exposure to cocaine, and the resulting inhibition may transiently reduce the tonic inhibitory influence of the VTA input on NAcCo neurons. Notably, the PPR of VTA-to-NAcCo inhibitory synapses appeared to exhibit developmental regulation; it increased when the animal became older (saline withdrawal day 1 vs. day 21).

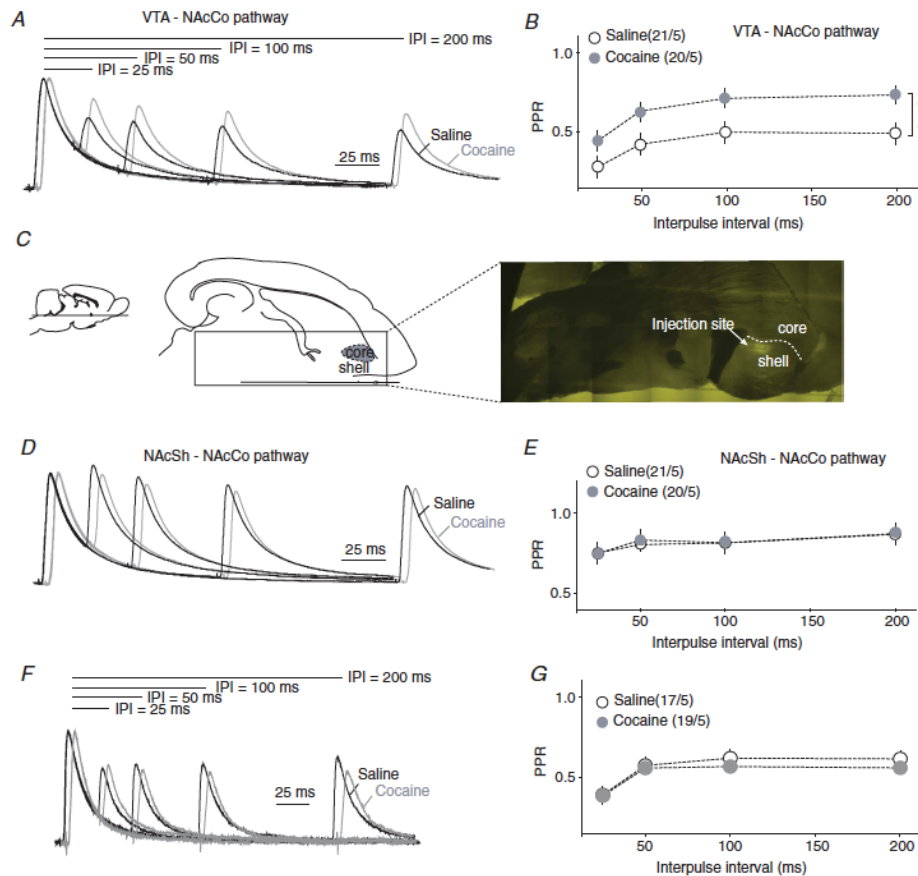


Figure 5. Presynaptic inhibition was detected at VTA-to-NAcCo inhibitory synapses after cocaine administration.

A. Example IPSCs elicited in the rat VTA-to-NAcCo synapses after 1 day of withdrawal by paired-pulse optical stimulations with different interpulse intervals (25, 50, 100 and 200 ms).

B. Summarized results showing that the PPRs of VTA-to-NAcCo inhibitory synapses were significantly increased after 1 day of withdrawal from repeated i.p. injections of cocaine.

C. Diagrams and image showing that intra-NAcSh injection of AAV2-ChR2Y resulted in the expression of fluorescent signals in the NAcSh.

D. Example IPSCs elicited at NAcSh-to-NAcCo synapses by paired-pulse stimulations with different interpulse intervals (25, 50, 100 and 200 ms) in NAcCo MSNs from saline and cocaine-treated rats.

E. Summarized results showing that the PPRs of NAcSh-to-NAcCo inhibitory synapses were not altered significantly after 1 day of withdrawal from repeated i.p. injections of cocaine.

F. Example IPSCs elicited at rat VTA-to-NAcCo synapses after 21 days of withdrawal by paired-pulse stimulations with different interpulse intervals (25, 50, 100 and 200 ms).

G. Summarized results showing that the PPRs of NAcSh-to-NAcCo inhibitory synapses were not altered significantly after 21 days of withdrawal from repeated i.p. injections of cocaine. $**P < 0.01$.

Typically, an increase in the PPR suggests decreased release probability in VTA-to-NAcCo fast inhibitory synaptic transmission. However, alterations in the postsynaptic receptor composition and/or receptor functionality may also contribute to the altered PPR (Heine et al., 2008). We therefore attempted to detect cocaine-induced postsynaptic alterations within this projection. At the postsynaptic membrane of GABAergic synapses, alterations in the subunit composition, typically the α subunits of GABA_ARs are a common form of cellular adaptation. Following exposure to cocaine, the whole-brain level of α 1 subunits is reduced (Koobs, 2001), which can be replaced by α 2/3 subunits. The switch from α 1 to α 2/3 subunits is accompanied by an elongation of the decay of IPSCs. In an attempt to determine potential cocaine-induced subunit switches in postsynaptic GABA_ARs at VTA-to-NAcCo inhibitory synapses, we first measured the time that elapsed from the IPSC peak to one-third of the peak amplitude ($T_{1/3}$) as a measure of the decay kinetics (Fig. 6A). $T_{1/3}$ at VTA-to-NAcCo inhibitory synapses was not altered significantly after cocaine exposure (in ms: Saline, 16.71 ± 2.08 , $n/m=4/2$; Cocaine, 17.10 ± 1.21 , $n/m=7/3$; $p=0.77$, t test). To verify this result, we next took a pharmacological approach. We superfused the slices with L838417 (100 nM), a compound that selectively enhances α 2/3-containing GABA_ARs and has a minimal effect on α 1-containing GABA_ARs at a concentration of 100 nM (Gross et al., 2011; Mathiasen, Rodgers, & Mirza, 2007; Mckernan et al., 2000; Morris, Dawson, Reynolds, Atack, & Stephens, 2006). Thus, if α 2/3 subunits were up-regulated by cocaine exposure, application of L838417 should increase the decay kinetics of IPSCs to a higher degree in cocaine-treated animals. However, upon application of L838417, $T_{1/3}$ was prolonged by very similar degrees in saline- and cocaine-treated animals ($F_{(1,18)}=0.10$, $p=0.75$, two-factor ANOVA; Fig. 6A), suggesting a lack of cocaine-induced effect on GABA_AR subunit composition. We then tested whether there was any cocaine-induced alteration in the subunit composition of GABA_ARs in

NACCo MSNs at the overall level by measuring the decay kinetics of spontaneous IPSCs that were presumably generated from all inhibitory synaptic inputs (evoked by electrical stimulation). Again, no significant alterations were detected (Saline, 11.41 ± 0.40 , $n/m=30/9$; Cocaine, 11.49 ± 0.29 , $n/m=34/7$; $p=0.20$, t test; Fig. 6B, C). We then made an additional attempt to examine whether the amplitude of postsynaptic responsiveness was altered at VTA-to-NACCo inhibitory synapses following exposure to cocaine. This can be tested by measuring the amplitude of miniature IPSCs, which reflects the quantal size of the synapse (magnitude of postsynaptic responsiveness to a single released vesicle). To functionally isolate the miniature IPSCs from the VTA-to-NACCo pathway, we evoked VTA-to-NACCo IPSCs in the presence of strontium (Sr^{2+}) (5 mM) to replace extracellular calcium (Ca^{2+}) and magnesium (Mg^{2+}). This replacement has been shown to desynchronize single evoked IPSCs and to produce a train of quantal events at the tail of individual evoked IPSCs. The asynchronous release occurs preferentially at terminals that have recently undergone synchronous release (e.g. evoked release), thus creating a time window to enrich miniature IPSC-like events from the recently activated synapses (Matsui & Jahr, 2003; Xu-Friedman & Regehr, 2000). As shown in Fig. 6D, we were able to evoke asynchronous release by light stimulations at VTA-to-NACCo synapses. Furthermore, the amplitude of these presumed miniature IPSCs was not altered in cocaine-exposed animals (Fig. 6E), suggesting a lack of cocaine-induced change in postsynaptic responsiveness at VTA-to-NACCo inhibitory synapses. Taken together, if potential postsynaptic alterations are excluded, the increased PPR probably reflects a cocaine-induced presynaptic inhibition of VTA-to-NACCo inhibitory synaptic transmission.

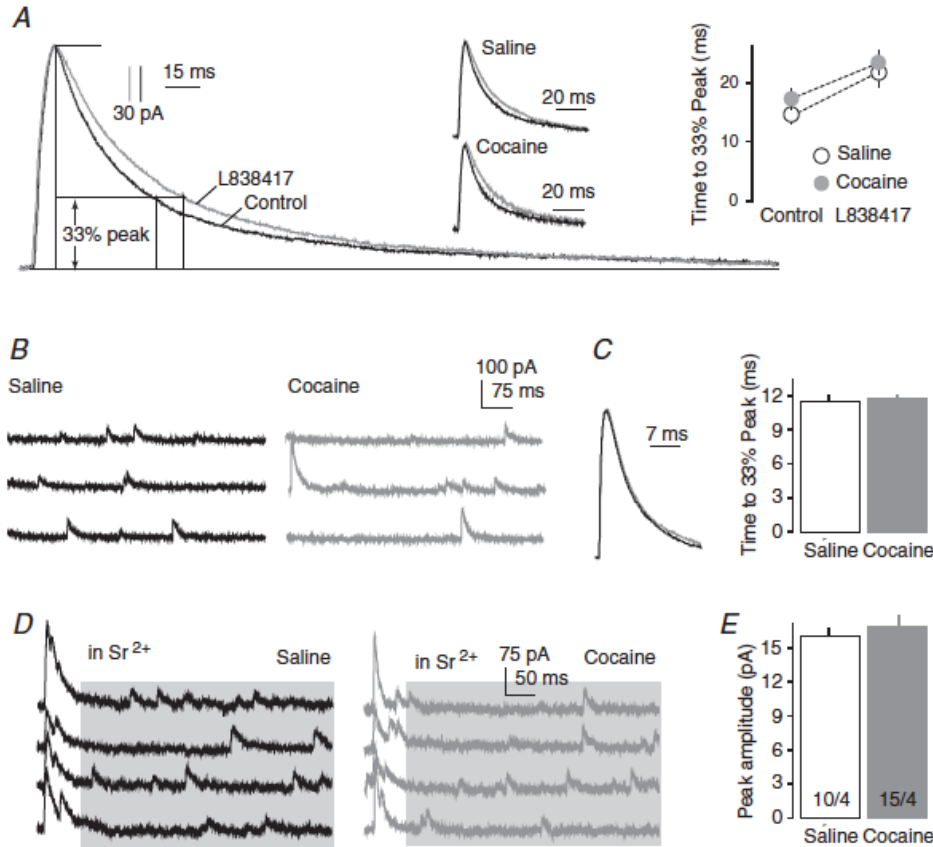


Figure 6. Cocaine-induced postsynaptic alterations in VTA-to-NAcCo inhibitory synapses were not detected.

A. Examples from control animal and summarized results showing that the decay kinetics of IPSCs recorded from the VTA-to-NAcCo projection were not altered significantly after repeated cocaine administration, and application of L838417 prolonged the decay of IPSCs to a similar degree between saline- and cocaine-treated rats (on withdrawal day 1).

B. Example spontaneous IPSCs in NAcCo MSNs from saline- and cocaine-treated rats.

C. Summarized results showing that the decay kinetics of spontaneous IPSCs in NAcCo MSNs were not altered significantly after 1 day of withdrawal from repeated I.P. injections of cocaine.

D. Examples showing that, in the presence of Sr²⁺, asynchronous IPSCs (in shaded area) were recorded following evoked IPSCs from VTA-to-NAcCo inhibitory synapses (via optical stimulation; events before the shaded area). These asynchronous IPSCs are largely attributable to the same set of synapses (i.e. VTA-to-NAcCo synapses) that mediate the evoked IPSCs.

E. Summarized results showing a lack of difference in the amplitude of asynchronous IPSCs from VTA-to-NAcCo inhibitory synapses between saline- and cocaine-exposed rats (on withdrawal day 1).

2.4 DISCUSSION

Interwoven with mesolimbic DA projection, fast excitatory and inhibitory synaptic inputs from the VTA to NAc may critically contribute to emotional and motivational responses (Birgner et al., 2010; Steffensen et al., 1998). VTA-to-NAc glutamatergic and GABAergic transmissions have been identified, but their cellular properties remain largely elusive.

Excitatory VTA-to-NAcCo synaptic transmission

The VTA-to-NAc excitatory synaptic inputs may originate from VTA DAergic neurons that co-release glutamate, from a small portion of glutamatergic neurons or from other neuronal types in the VTA (Chuhma et al., 2004; Hnasko et al., 2010; Joyce & Rayport, 2000; Lavin et al., 2005; Nair-Roberts et al., 2008; Stuber et al., 2010; Sulzer et al., 1998; Tecuapetla et al., 2010). Studies using genetically modified mice have demonstrated that the VTA glutamatergic output regulates drug-induced locomotor and seeking responses, probably via regulation of the DA dynamics within the NAc (Alsiö et al., 2011; Birgner et al., 2010; Hnasko et al., 2010). Our study detected a modest cocaine-induced presynaptic alteration in VTA-to-NAc excitatory synapses. However, we did not detect cocaine-induced alterations in the postsynaptic responses, which were assessed by the relative weight of AMPAR- and NMDAR-mediated EPSCs rather than their absolute values. As such, the results cannot rule out the possibility of postsynaptic changes, such as a parallel up- or down-regulation of postsynaptic AMPARs and NMDARs. This parallel scaling may happen during the generation of new mature synapses or the degeneration of pre-existing synapses. Furthermore, although VTA-to-NAc excitatory synapses contribute to the depolarization of NAc MSNs, their contribution is not likely to be predominant compared with other excitatory

synaptic inputs. Rather, a potentially more significant role of VTA-to-NAc excitatory synaptic transmission may lie in the regulation of VTA-to-NAc DA terminals, their release of DA or other synaptic inputs to the NAc, as suggested previously (Alsiö et al., 2011; Hnasko et al., 2010; Tecuapetla et al., 2010).

Inhibitory VTA-to-NAcCo synaptic transmission

GABAergic neurons constitute about 30% of VTA neurons; they receive synaptic projections from the same or approximate brain regions that innervate DAergic neurons, project to the same brain regions as DAergic neurons and are activated simultaneously with DAergic neurons upon reward stimulation (Carr & Sesack, 2000; Kiyatkin & Rebec, 2001; Nair-Roberts et al., 2008; Steffensen, Lee, Stobbs, & Henriksen, 2001). However, it remains unclear whether exposure to cocaine affects VTA-to-NAcCo inhibitory synapses. Our results show that VTA-to-NAcCo inhibitory synapses exhibit a higher presynaptic release probability than NAcSh-to-NAcCo input. Given that VTA GABAergic neurons tonically spike at ~20 Hz in vivo (Steffensen et al., 1998), a frequency much higher than NAcSh MSNs (O'Donnell & Grace, 1995; O'Donnell et al., 1999), VTA-to-NAcCo inhibitory synapses may contribute substantially to the basal inhibition of NAcCo MSNs. As such, decreased presynaptic release probability of these synapses following cocaine exposure may undermine this inhibition, potentially resetting the balance between 'distant' and 'local' inhibitory controls of NAcCo MSNs. Although it has been reported that very few VTA GABAergic neurons project to NAc MSNs (M. T. Brown et al., 2012), stable VTA-originated IPSCs were detected in our and previous studies (van Zessen et al., 2012). Our recordings were primarily made in the caudal NAcCo, and the discrepant results may reflect the fact that NAcCo MSNs within different subregions possess different synaptic connections.

Effects of cocaine and cocaine withdrawal

Our results suggest that cocaine-induced alterations in VTA-to-NAc excitatory transmission are different from the other excitatory projections to the NAc. After 1 day of withdrawal, we did not detect cocaine-induced alterations in either the PPR or AMPAR/NMDAR ratio (Fig. 2), suggesting the lack of pre- or postsynaptic alterations. However, randomly sampled excitatory synapses on NAcCo neurons do not show presynaptic alterations following similar cocaine regimens (Curcio et al., 2013; Dobi, Seabold, Christensen, Bock, & Alvarez, 2011), but exhibit an increase in the AMPAR/NMDAR ratio in a recent study (Curcio et al., 2013) (See Discussion). In comparison, glutamatergic synapses onto NAcSh neurons are more dynamically regulated following similar cocaine regimens. We have shown previously that the presynaptic release probability of medial PFC-to-NAcSh excitatory synapses is increased at this withdrawal time point (Suska et al., 2013). Furthermore, randomly sampled excitatory synapses in the NAcSh exhibit a decreased AMPAR/NMDAR ratio (Kourrich et al., 2007; Mameli et al., 2009). This decrease can be mediated by a potential down-regulation of synaptic AMPARs (Boudreau et al., 2007; Boudreau & Wolf, 2005) or an up-regulation of NMDARs (T. E. Brown et al., 2011; Y. H. Huang et al., 2009). Thus, at this withdrawal time point, pre- or postsynaptic alterations are detected in some other excitatory afferents, but not in the VTA-to-NAc projection.

On withdrawal day 1, when no changes were detected at the glutamatergic transmission at VTA-to-NAcCo synapses, there was a significant suppression of GABAergic transmission within this projection (Fig. 5A, B). By contrast, on withdrawal day 21, as GABAergic transmission returned to control levels (Fig. 5F, G), glutamatergic transmission showed presynaptic enhancement (Fig. 2G, H). Thus, the differential pre- synaptic alterations may shift the inhibitory–

excitatory balance within the VTA-to-NAcCo projection during different stages of cocaine withdrawal, and may thus contribute to the time-dependent withdrawal-associated behavioral alterations. An additional factor that may tip the balance of NAc output is through the differential innervations of subpopulations of NAc MSNs. For example, DA D1 and D2 receptor-expressing MSNs in the NAc have been shown to differ in their cellular responses to addictive drugs and could play opposing roles in cocaine-associated behaviors (Lobo et al., 2010; Smith, Lobo, Spencer, & Kalivas, 2013). Although these two types of neuron may share similar synaptic inputs, the sub-cellular connectivity patterns may be biased to convey pathway-specific signaling (MacAskill, Little, Cassel, & Carter, 2012). It remains to be determined whether D1-and D2-expressing MSNs in the NAc also receive differential innervations from the VTA DAergic, glutamatergic and GABAergic transmissions, and whether the innervation patterns are altered by cocaine experience.

In summary, the present study characterized some basic electrophysiological properties of VTA-to-NAcCo fast excitatory and inhibitory synapses, and detected cocaine-induced presynaptic adaptations within these afferents. These results may provide a knowledge and technical basis with which to explore the role of VTA-to-NAc fast synaptic transmission in cocaine-induced emotional and motivational alterations.

3.0 THE REGULATION OF VTA-TO-NAC GABAERGIC TRANSMISSION BY CO-RELEASED DOPAMINE AND THE EFFECT OF COCAINE EXPOSURE²

3.1 INTRODUCTION

Synaptic projections from VTA to the NAc are essential for emotional and motivational responses (Wise, 1987). The VTA-to-NAc pathway has parallel DAergic and GABAergic projections, and these projections are often concurrently activated upon the same emotional/motivational stimuli (Carr & Sesack, 2000; Steffensen et al., 1998). This “intimate” anatomical and physiological partnership suggests that the VTA-to-NAc DAergic and GABAergic projections may exhibit unique interactions upon their co-activation during emotional and motivational responses. We did not observe the effect from concurrent DA signaling to the VTA-to-NAcCo GABAergic projection in last Chapter. Here we investigate the interaction by focusing on LTD. Using optogenetic techniques combined with electrophysiological manipulations, we demonstrated that activation of VTA-to-NAc DAergic projections triggers LTD at GABAergic synapses within this pathway. We further demonstrated that this LTD is triggered by heterosynaptic DA and is abolished after withdrawal from cocaine exposure. By pharmacological manipulations of DA receptors, extensive prior results establish that DA is a modulator of plasticity. Our results indicate that action-potential-driven DA release is also a trigger of plasticity. This form of LTD relieves NAc neurons from inhibition upon receipt of phasic input from DA

² The work presented in Chapter 3 is from “Dopamine triggers heterosynaptic plasticity.” M. Ishikawa*, M. Otaka*, Y.H. Huang, P.A. Neumann, B.D. Winters, A.A. Grace, O.M. Schluter, and Y. Dong. 2013. *J Neurosci*, 33(16), 6759-6765.

neurons that has been shown to be triggered by reward or reward-associated cues (Fiorillo, Tobler, & Schultz, 2003). Disruption of this LTD may contribute to the rigidity in reward-associated responses that occur during cocaine withdrawal.

3.2 MATERIALS AND METHODS

Viral vectors

ChR2 fused to Venus (AAVChR2Y; Addgene plasmid 20071) or mCherry (AAV-flexed-ChR2R; Addgene plasmid 18916) were expressed from AAV2 with AAV2 internal repeats (Atasoy et al., 2008; Petreanu et al., 2009; Suska et al., 2013). AAV-flexed ChR2R expression was induced specifically in Cre-recombinase-expressing neurons, in which the inverted expression cassette was flipped (Atasoy et al., 2008). As previously described, flexed virus was used in Gad-Cre or TH-Cre mouse lines.

Animal use, stereotaxic injections, and cocaine administration

Male Sprague-Dawley rats (Simonsen, CA, USA), the Gad2-IRES-Cre mouse line (Gad2^{tm2(cre)Zjh}; The Jackson Laboratory), and the TH-IRES-Cre mouse line (B6.129X1-Th^{tm1(cre)Te}/Kieg; European Mouse Mutant Archive) (Lindeberg et al., 2004) were used. Intra-VTA viral injections were performed when animals were at the age of postnatal 26–34 days. Virus was delivered stereotaxically in animals anesthetized with a mixture of ketamine/xylazine (50/6 mg/kg) (Y. H. Huang, Ishikawa, et al., 2011). A 28-gauge needle was used to bilaterally inject 1 μ l (0.2 μ l/min) of the virus solution into the VTA. Experiments were performed about 3 weeks after viral injection. After recovery, rats received i.p. injections of cocaine (15 mg/kg/day in saline for 5

consecutive days) or saline (0.1 ml/100 g) in a novel environment (T. E. Brown et al., 2011; Ishikawa et al., 2009; Mu et al., 2010). Animals were placed back in the home cage for withdrawal.

NAc slice preparations and electrophysiology

As described previously, the animals were decapitated after being given isoflurane anesthesia. The brain was sliced in 4 °C cutting solution containing the following (in mM): 135 N-methyl-D-glucamine, 1 KCl, 1.2 KH₂PO₄, 0.5 CaCl₂, 1.5 MgCl₂, 20 choline-HCO₃, and 10 glucose, saturated with 95% O₂/5% CO₂, pH adjusted to 7.4. Slices were incubated in aCSF containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose, saturated with 95% O₂/5% CO₂ at 37 °C for 30 min and then at room temperature before experimentation. During recordings, slices were superfused with aCSF at 31–33°C. Recordings were made with electrodes (3–5 MΩ) filled with (in mM): 140 CsCH₃O₃S, 5 TEA-Cl, 0.4 EGTA, 20 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP, and 1 QX-314, pH 7.3.

To evoke EPSCs/IPSCs, axons expressing ChR2 were stimulated by 473 nm laser (IkeCool) coupled to a 62.5 μm optic fiber. The optical stimulation was given with 0.05–1 ms duration at 0.1 Hz. EPSCs were recorded at -70 mV and IPSCs at +10 mV. To induce presynaptically expressed LTD of IPSCs, a modest protocol (5 Hz×10 min) was shown to be sufficient (Chiu, Puente, Grandes, & Castillo, 2010). In our experiments, an even more modest protocol (2 Hz×10 min) could reliably induce LTD at VTA-to-NAcCo inhibitory synapses.

For all recordings, series resistance was 8–14 MΩ and was left uncompensated. Series resistance was monitored continuously, and a change more than 20% was not accepted for data analysis. Synaptic currents were recorded with a MultiClamp 700B amplifier (Molecular Devices) filtered at 2.6–3 kHz and digitized at 20 kHz.

Drugs

Chemicals were purchased from Tocris Bioscience (Bristol, UK) and Sigma-Aldrich (St Louis, MO, USA).

Data analysis and statistics

All results are shown as mean \pm SEM. Statistical significance was assessed using either one- or two-factor ANOVA with Bonferroni posttests or two-tailed t test. One to four cells were recorded from each animal. The total numbers of cells/animals are presented as “n/m.” Cell-based statistics were performed for all results.

3.3 RESULTS

To record IPSCs from the VTA to NAcCo MSNs, we used viral-mediated gene transfer with AAV to express ChR2Y in the VTA *in vivo*. Three weeks later, we obtained horizontal brain slices within which the VTA exhibited yellow fluorescent signals, indicating the expression of ChR2 (Fig. 7A). Within the same slices, ChR2Y positive neural fibers were observed projecting from the VTA to the forebrain including the NAc (Fig. 7A). By laser-light mediated activation of ChR2 (0.05–1 ms duration, $\lambda=473$ nm), VTA-to-NAcCo GABAergic and DAergic/glutamatergic synaptic transmissions were activated simultaneously (Fig. 7B). GABAergic transmission was recorded at +10 mV, near the reversal potential of glutamatergic transmission. IPSCs were detected in most (37/46) recorded NAcCo neurons (Fig. 7C); neurons with no response or slow inward current were excluded from subsequent experiments. We focused on LTD at VTA-to-NAcCo

GABAergic synapses, a form of synaptic plasticity that may relieve NAcCo MSNs from basal inhibition of their output. We optimized a low frequency stimulation (LFS; 2 Hz×10 min) induction protocol, delivery of which via optical stimulation induced a reliable LTD at VTA-to-NAcCo inhibitory synapses ($p=0.01$, t test; Fig. 7D). Two prominent features of this LTD were: (1) it continued developing during the 50 min recording, a property similar to LTD of IPSCs in other brain regions (Chiu et al., 2010; Heifets, Chevaleyre, & Castillo, 2008; Morishita & Sastry, 1996) and (2) it was accompanied by an increase in the paired-pulse ratio (PPR), suggesting a presynaptic site of expression ($p=0.05$, paired t test; Fig. 7D–F). Under this experimental setup, both GABAergic and DAergic/glutamatergic signaling was activated during LTD induction, of which the GABA_B- (Chalifoux & Carter, 2011) and DA/glutamate-signaling pathways (Castillo et al., 2011) have been shown to regulate inhibitory synaptic transmission presynaptically. Our subsequent results did not suggest a predominant role of GABA_B signaling; inhibition of GABA_B receptors by superfusion of GABA_B-receptor-selective antagonist CGP55845 (10 μ M) did not prevent LFS-induced LTD ($F_{(3,24)}=20.79$, $p<0.01$, one factor ANOVA; $p<0.01$, CGP55845 prestimulation vs CGP55845 poststimulation; $p=1.0$, control poststimulation vs CGP55845 poststimulation, Bonferroni posttests; Fig. 7G). However, inhibition of GABA_B receptors abolished the continuously developing feature of this LTD (Fig. 7G). Upon activation of DAergic terminals, it is possible that glutamate is co-released to activate postsynaptic group I mGluRs. This may induce a release of endocannabinoids, triggering presynaptically expressed LTD (Castillo et al., 2011). However, coinhibition of mGluR1 and 5 by superfusion of their selective antagonists LY367385 (100 μ M) and MPEP (10 μ M) did not prevent this LTD ($F_{(3,24)}=17.4$, $p<0.01$, one-factor ANOVA; $p<0.01$, prestimulation vs poststimulation in antagonists; $p=1.0$, control poststimulation vs antagonists poststimulation, Bonferroni posttests; Fig. 7H). In contrast to GABA_B and mGluRs,

inhibition of DA D1Rs by SCH23390 (1 μ M) prevented the induction of LTD ($F_{(3,20)}=30.06$, $p<0.01$, one-factor ANOVA; $p=1.0$, SCH23390 prestimulation vs SCH23390 poststimulation; $p<0.01$, control poststimulation vs SCH23390 poststimulation, Bonferroni posttests; Fig. 7I). Furthermore, inhibition of DA D2Rs by eticlopride (3 μ M) did not completely prevent LTD ($F_{(3,18)}=12.56$, $p<0.01$, one-factor ANOVA; $p=0.20$, control poststimulation vs eticlopride poststimulation; $p=0.28$, eticlopride prestimulation vs eticlopride poststimulation, Bonferroni posttests; Fig. 7J). These results suggest that induction of this LTD required DA signaling, which was likely a novel form of heterosynaptic plasticity involving the parallel VTA-to-NAcCo DAergic terminals; the heterosynaptic nature of this plasticity is likely mediated by nearby DAergic terminals via local heterosynaptic transmission or “spillover” of DA from distal DAergic terminals (Fig. 7K).

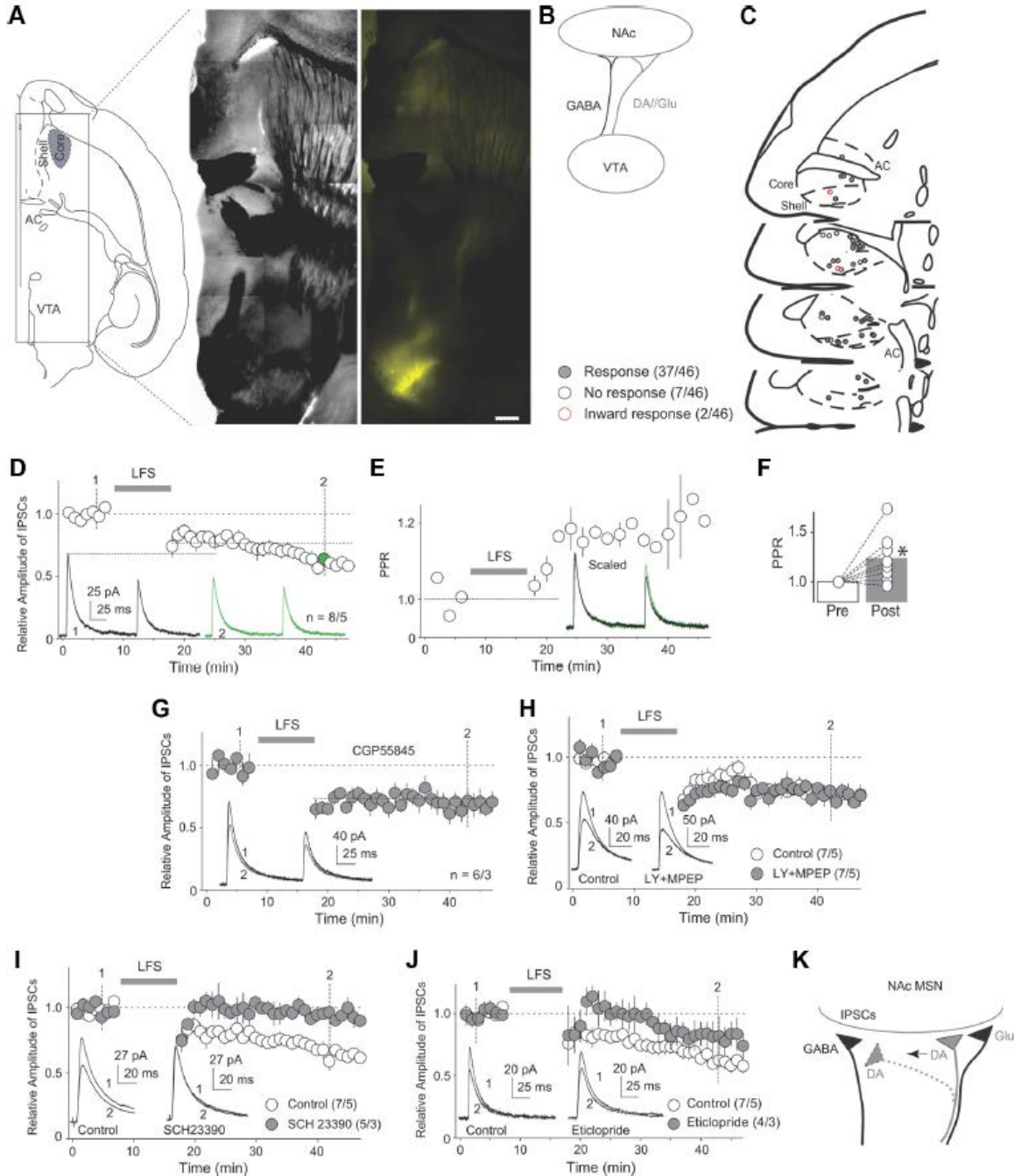


Figure 7. LTD at VTA-to-NAcCo GABAergic Synapse.

A. Diagrams and images showing that intra-VTA injection of ChR2-expressing virus resulted in expression of ChR2 in fibers within the NAcCo in a horizontal brain slice. Scale bar, 500 μm .

B. Diagram showing that in the rat preparation, VTA-to-NAcCo projections include both GABAergic and DAergic/glutamatergic fibers.

C. Diagrams showing locations of recorded neurons exhibiting IPSCs (responses), no responses,

and inward synaptic currents upon optical stimulations.

D. Summarized results showing LTD induced by an LFS protocol at VTA-to-NAcCo GABAergic synapses. Inset: Example of IPSCs before and after LTD induction. Numbers (1 and 2) indicate the time points when the example IPSCs were taken.

E. Time course of the PPR of IPSCs in Figure D. Inset: Scaled IPSCs of the examples shown in Figure D.

F. Summarized results showing that this LTD was accompanied by an increase in the PPR.

G. Summarized results showing that this LTD was not prevented in the presence of the GABA_B-receptor-selective antagonist CGP55845.

H. Summarized results showing that this LTD was not prevented in the presence of mGluR1- and mGluR5-selective antagonists LY367385 and MPEP.

I. Summarized results showing that this LTD was prevented by the DA D1R-selective antagonist SCH23390.

J. Summarized results showing that this LTD was not prevented by the DA D2R-selective antagonist eticlopride.

K. Diagram showing that the parallel DAergic terminals may provide heterosynaptic modulation of VTA-to-NAcCo GABAergic transmission. * $p < 0.05$.

We next tested this possibility by studying VTA-to-NAcCo GABAergic and DAergic/glutamatergic inputs using optogenetic tools. To isolate GABAergic input, we used GAD-Cre mice (Taniguchi et al., 2011), in which we injected an inducible AAV-ChR2R (flexed-ChR2R) into the VTA. Therefore, only GABAergic neurons, but not DAergic/glutamatergic neurons, expressed ChR2R and projected ChR2R-expressing fibers to the NAcCo (Fig. 8A,B). Although it has been reported that very few VTA GABAergic neurons project to NAc MSNs (M. T. Brown et al., 2012), stable IPSCs were detected in our study. Given that our recordings were focused on the caudal NAcCo (Fig. 8C), the discrepant results may reflect that NAcCo neurons within different subregions possess different synaptic connections. Nonetheless, the efficacy was assessed electrophysiologically. Briefly, because DA is co-released with glutamate at these terminals (Lavin et al., 2005; Nair-Roberts et al., 2008; Stuber et al., 2010; Sulzer et al., 1998), activation of DAergic fibers can be detected operationally by the presence of EPSCs (Fig. 8C). In rat brain slices with intra-VTA injection of regular AAVChR2Y, laser stimulation elicited both IPSCs (PTX sensitive) and EPSCs (NBQX sensitive) in most of the recorded neurons (35/36). In contrast, in GAD-Cre mice receiving AAV-flexed ChR2R, NBQX sensitive currents were rarely observed (4/30; Fig. 8C), suggesting that the VTA GABAergic projection was isolated successfully. Using this approach, we found that laser-mediated LFS did not induce LTD at VTA-to-NAcCo inhibitory synapses ($p=0.09$, paired t test; Fig. 8D), indicating that activation of GABAergic transmission alone did not induce LTD and that the LTD is likely heterosynaptic in nature.

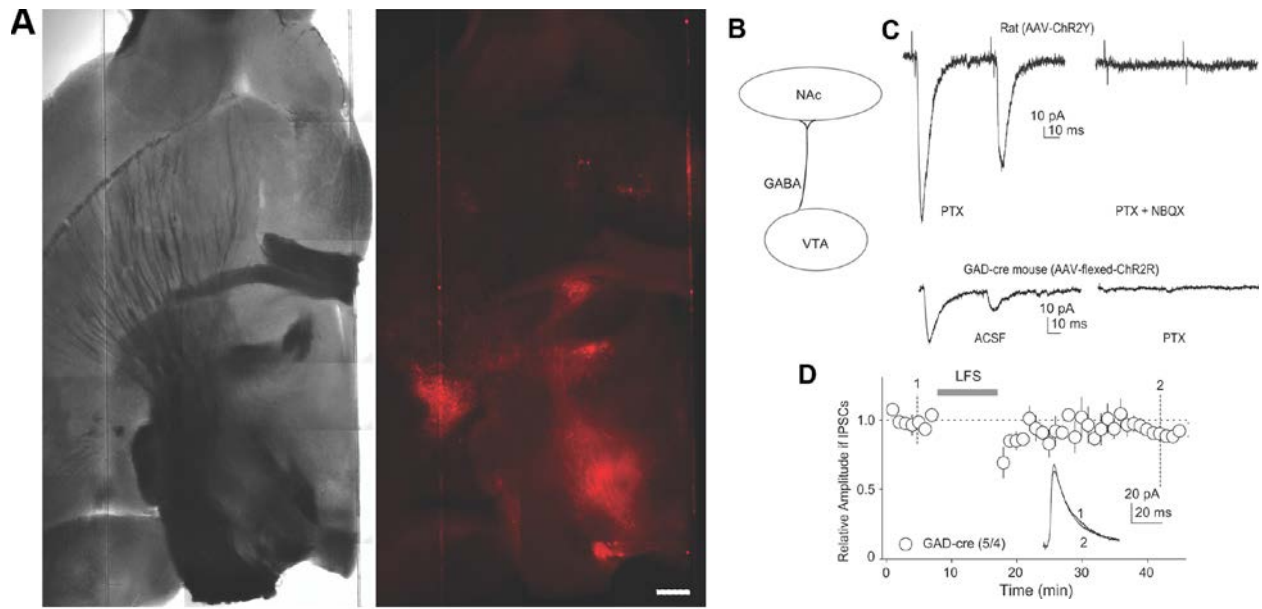


Figure 8. LTD at VTA-to-NAcCo inhibitory synapses could not be induced by activation of these synapses alone.

A. Images showing that intra-VTA injection of flexed ChR2R resulted in expression of ChR2R (red fluorescent signals) in fibers within the NAcCo in a horizontal brain slice from a GAD-Cre mouse. Scale bar, 500 μm .

B. Diagram showing that with this optogenetic approach, optical stimulation activated VTA-to-NAcCo GABAergic transmission selectively.

C. Example traces showing that in brain slices prepared from rats (top) in which ChR2-AAV was injected, in addition to IPSCs, optical stimulation often elicited EPSCs (recorded at -70 mV, sensitive to NBQX), suggesting an infection of VTA-to-NAcCo DAergic/glutamatergic projection. In brain slices prepared from GAD-cre mice in which ChR2-flexed AAV was injected (bottom), optical stimulation rarely elicited EPSCs, suggesting that only the GABAergic projection was infected.

D. Summarized results showing that when the GABAergic VTA-to-NAcCo projection was stimulated selectively (flexed ChR2R in GAD-cre mice), LFS failed to induce LTD. Inset: Example of IPSCs before and after LFS stimulation.

To determine whether heterosynaptic DA signaling was the key, we combined optogenetic techniques with the minimal stimulation assay. We used TH-Cre mice (Lindeberg et al., 2004) that received intra-VTA injection of AAV-flexed ChR2R. Therefore, only DAergic neurons expressed ChR2R and projected ChR2R-expressing fibers to the NAcCo (Fig. 9A). The DAergic/glutamatergic specificity of this approach was confirmed by the consistent appearance of EPSCs in NAcCo neurons upon optical stimulation (11/12). In addition, as described previously (Tritsch et al., 2012), activation of VTA-to-NAcCo DAergic/glutamatergic terminals also elicited PTX sensitive (100 μ M) IPSCs in more than half (23/33) of recorded NAcCo neurons (Fig. 9B). However, IPSCs evoked from VTA-to-NAcCo DAergic projection exhibited a significant rundown by ~50% within the first minute and declined by ~90% within 5 min (Fig. 9C). This decaying kinetics is consistent with the notion that GABA released from the VTA-to-NAcCo DAergic projection is “packaged” together with dopamine and released from the same vesicles (Tritsch et al., 2012). Because the baseline of our LTD experiments was typically established after 10 min of recording, it is not likely that GABA release from the DAergic projection contributed significantly to either the baseline IPSCs or IPSCs after LTD shown in Figure 1. Furthermore, these results do not support a role for GABAergic signaling in the induction of this LTD (Fig. 7G, Fig. 8D). Therefore, we did not focus on this atypical transmission in the subsequent assays. Within the NAc slices containing ChR2R-expressing DAergic fibers, we established a minimal stimulation procedure (Fig. 9D). Through an electrical stimulation electrode, we applied weak stimulations such that only a small number of inhibitory synapses were included (recorded at around the reversal potential of EPSCs). Although this approach allowed for the examination of individual inhibitory synapses, these synapses did not necessarily originate from the VTA. In the example shown in Figure 9E, two functional synapses were included, which should produce three

types of postsynaptic responses throughout the trial: (1) failures, when both synapses fail to release neurotransmitters; (2) small responses, when one of the two synapses is successfully activated (depending on the actual properties and locations of these two putative synapses, small responses may not be identical); and (3) large responses, when both synapses are activated simultaneously. The incidence of failures, small responses, or large responses is determined by the presynaptic release probability.

This technique offers two detectable parameters with which to assess the presynaptic or postsynaptic expression mechanisms of LTD: the failure rate, for which a decreased release probability would be accompanied by an increased rate of failures and a decreased rate of large responses, and the amplitude of small responses, which is likely mediated by a single unitary response. We recorded IPSCs from NAcCo neurons evoked by minimal stimulation and then applied LFS to the VTA-to-NAcCo DAergic/glutamatergic input using optical stimulations. Our results show that the overall amplitude of IPSCs (including responses and failures) was decreased in an LTD-like manner ($p=0.01$, paired t test; Fig. 9F,G). This LTD was accompanied by an increased failure rate, suggesting a decrease in presynaptic release probability ($p=0.04$, paired t test; Fig. 9H). Furthermore, when the Gaussian distributions of all responses (from all recorded cells) were plotted before and after the LTD induction, it became apparent that the LTD procedure reduced the incidence of large responses without affecting the amplitude of small responses ($p=0.59$, paired t test; Fig. 9I-K). These results suggest that activation of heterosynaptic DA signaling is sufficient to induce presynaptically expressed LTD identified at VTA-to-NAcCo inhibitory synapses (Fig. 7).

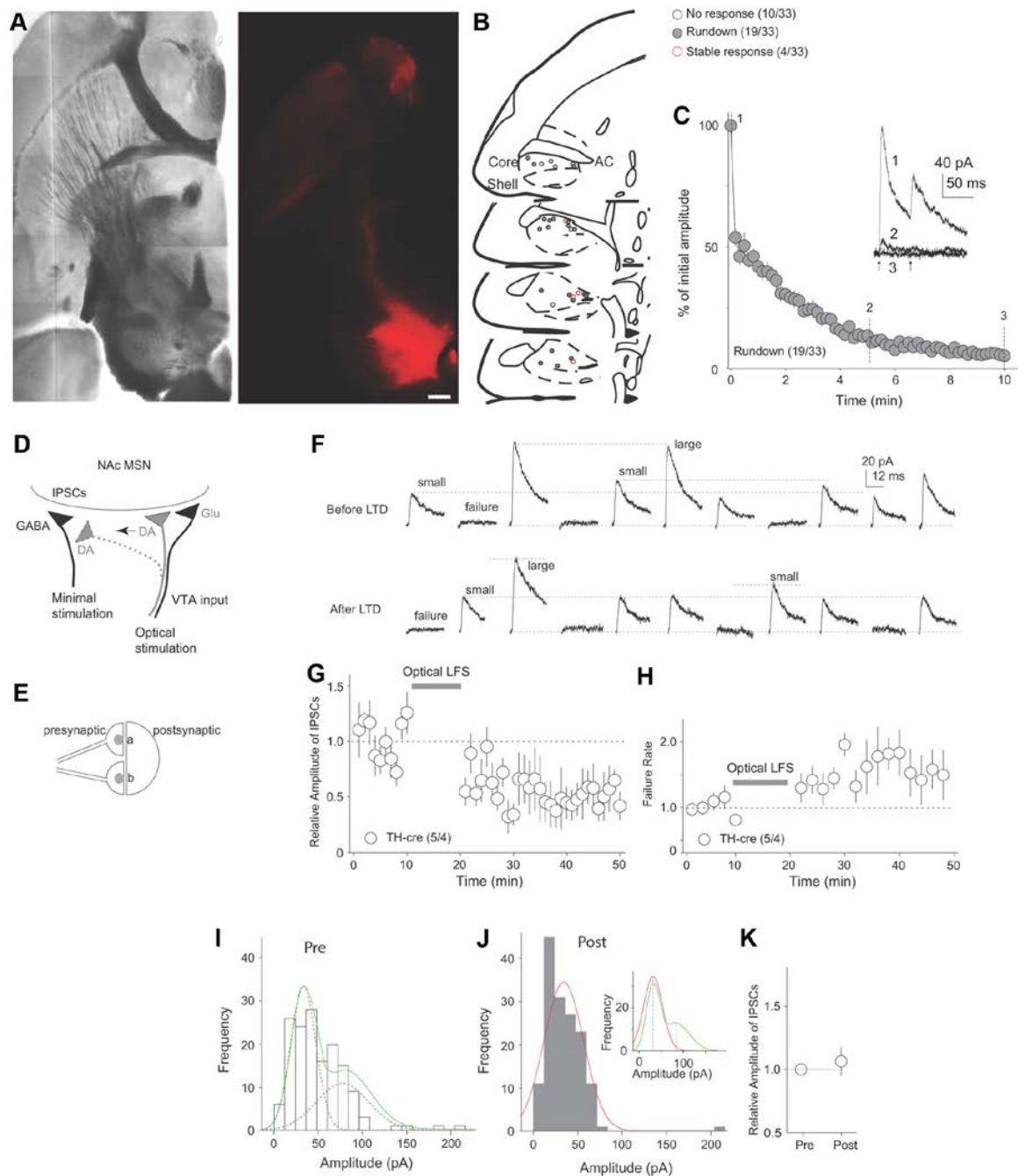


Figure 9. LTD by heterosynaptic dopamine.

A. Images showing that intra-VTA injection of flexed ChR2R resulted in expression of ChR2R (red fluorescent signals) in fibers within the NAcCo in a horizontal brain slice from a TH-cre mouse. Scale bar, 500 μ m.

B. Diagram showing the locations of recorded NAcCo neurons that exhibited no synaptic responses, IPSCs with significant rundown, and stable IPSCs in a subset of experiments.

C. Summarized results showing that IPSCs from VTA-to-NAcCo DAergic projections declined rapidly during recording. Stimulation frequency, 0.1 Hz.

D. Diagram showing the experimental setup for minimal stimulation assay in which GABAergic synaptic transmission was evoked by electrical stimulation in a minimal stimulation protocol and activation of DA signaling was achieved by optical stimulation.

E. Diagram showing an example of the minimal stimulation setup in which two synapses are included by the stimulation. Three types of postsynaptic responses can be generated: (1) failures, when both synapses fail to release neurotransmitter; (2) small responses, when one of the two synapses is activated successfully; and (3) large responses, when both synapses are activated simultaneously. The incidences of failures, small responses, or large responses are determined by the presynaptic release probability.

F. Example of IPSCs evoked by the minimal stimulation protocol in NAcCo neurons before and after the induction of LTD.

G. Summarized results showing that LTD was induced by the optically applied LFS (only dopaminergic fibers were activated by this LFS). All responses including failures were included in this summary.

H. Summarized results showing that the LTD was accompanied by an increase in the failure rate.

I. J. Summarized results showing that the LTD induction did not change the amplitude of small responses, but reduced the incidence of large responses. The amplitudes of IPSCs evoked by the minimal stimulation protocol were well fit by two-Gaussian distributions before LTD induction (**I**), presumably corresponding to the small and large responses, and one Gaussian distribution after LTD (**J**). Inset in Figure J shows that the amplitude of small responses did not change, whereas the incidence of large responses was reduced.

K. Summarized results showing that the amplitude of small responses was not altered by LTD induction.

By compromising the ability of synapses to undergo plastic changes, exposure to cocaine or other drugs of abuse could potentially reduce the flexibility of the brain during its response to emotional/motivational stimuli (Kasanetz et al., 2010). Consistent with this, we observed in rats that the LTD at VTA-to-NAcCo inhibitory synapses was minimally reduced after 1 d withdrawal after repeated exposure to cocaine ($F_{(3,24)}=36.24$, $p<0.01$, one-factor ANOVA; $p=0.39$, saline poststimulation vs cocaine poststimulation; $p<0.001$, cocaine prestimulation vs cocaine poststimulation, Bonferroni posttests; Fig. 10A), but largely abolished when the withdrawal time extended to 3 weeks ($F_{(3,28)}=21.38$, $p<0.01$, one-factor ANOVA; $p<0.01$, saline poststimulation vs cocaine poststimulation; $p=0.13$, cocaine prestimulation vs cocaine poststimulation, Bonferroni posttests; Fig. 10B). Note that the pattern of LTD was slightly altered in an age-dependent manner.

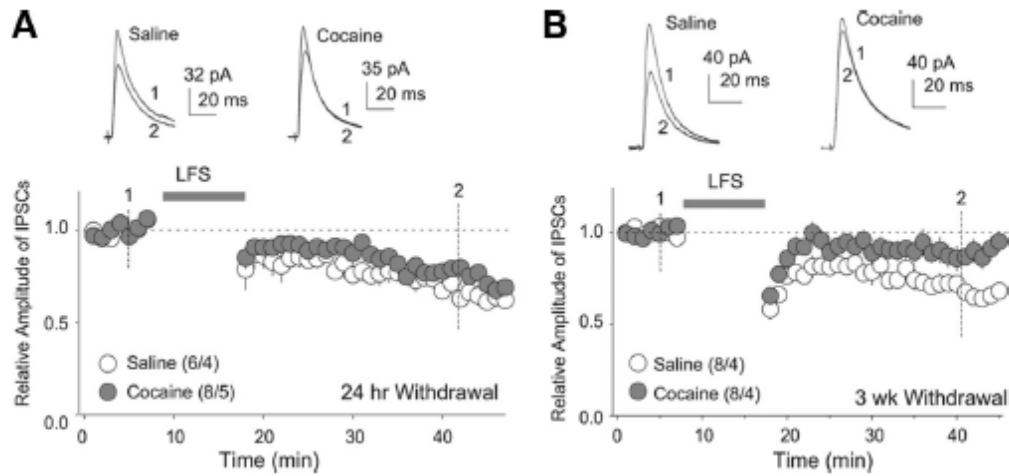


Figure 10. LTD at VTA-to-NAcCo inhibitory synapses is disrupted after cocaine withdrawal.

A. Summarized results showing that LTD at VTA-to-NAcCo inhibitory synapses was slightly attenuated after 1 d withdrawal from repeated i.p. injections of cocaine. Insets show examples of IPSCs at the time points indicated by the numbers.

B. Summarized results showing that LTD at VTA-to-NAcCo inhibitory synapses was significantly attenuated after a 3-week withdrawal from repeated i.p. injections of cocaine. Insets show examples of IPSCs at the time points indicated by the numbers.

3.4 DISCUSSION

GABAergic neurons constitute approximately one-third of the neuronal population of the VTA, receive synaptic projections from the same brain regions that innervate DAergic neurons, project together with DAergic neurons to the same brain regions, and are activated simultaneously with DAergic neurons upon reward stimulation (Carr & Sesack, 2000; Nair-Roberts et al., 2008; Steffensen et al., 1998). Despite such an “intimate” anatomic setup, little is known regarding how VTA DA and GABA transmissions interact. Our results demonstrate a form of LTD at the VTA-to-NAcCo GABAergic projection that is triggered by the parallel DAergic signaling. This heterosynaptic LTD was disrupted after withdrawal from cocaine administration. These findings may provide insight into understanding the mechanisms of reward- and cocaine-induced cellular and circuitry responses.

First, although experience-dependent plasticity at inhibitory synapses has been observed in many brain regions (Castillo et al., 2011), a strikingly different feature of the currently identified LTD is that it relies on heterosynaptic DAergic signaling. It has long been thought that DA acts as a neuromodulator but does not induce long-term synaptic plasticity directly (Calabresi, Picconi, Tozzi, & Di Filippo, 2007). Our study represents one of the first demonstrations that heterosynaptic DA signaling can trigger synaptic plasticity independently, unveiling another biological function of DA. This feature is particularly important for the physiological role of VTA-to-NAcCo GABAergic transmission. VTA GABAergic neurons spike continuously *in vivo* (Steffensen et al., 1998). This constant firing is translated into stable background inhibitory control of NAc neurons only if the firing does not induce adaptive changes, which based on our results is the case (Fig. 8). Furthermore, upon exposure to incentive stimuli, VTA DAergic neurons increase firing and the

resulting heterosynaptic DA signaling may induce incentive-contingent LTD of VTA-to-NAcCo inhibitory transmission. Indeed, based on the results in the minimal stimulation assay (Fig. 9), this DA-mediated heterosynaptic LTD may be a common feature for most GABAergic synapses on NAcCo neurons. With this heterosynaptic LTD, the incentive-elicited activation of the NAc can be regulated in a time-contingent manner.

Second, as mentioned above, if the LTD demonstrated here indeed serves as an efficient mechanism to relieve the basal inhibition of NAcCo MSNs upon incentive stimuli such that NAc neurons can engage in the processing of related emotional/motivational information, a conceivable consequence of the disruption of this LTD is that the activity pattern of VTA-to-NAcCo transmission is locked, resulting in compromised flexibility/capability of NAcCo to process incentive stimuli after cocaine withdrawal. This may correspond to several withdrawal symptoms that promote relapse: a rigid emotional state favorable to drug-taking, reduced responses to non-drug-related emotional and motivational stimuli, and persistent hypoactivity of NAc (Koob & Le Moal, 2006). The heterosynaptic LTD described herein suggests a novel role of DA as a plasticity trigger, and this unique interaction between DAergic and GABAergic signaling within the VTA-to-NAcCo projection may provide a new angle in exploring the structural and physiological basis of the mesocorticolimbic DA.

4.0 EXPOSURE TO COCAINE REGULATES INHIBITORY SYNAPTIC TRANSMISSION³

4.1 INTRODUCTION

The NAc functions to gate emotional and motivational arousals for behavioral output (Mogenson & Huang, 1973; Mogenson et al., 1980). Exposure to cocaine and other drugs of abuse induces cellular adaptations in NAc MSNs, and the resulting functional alterations of the NAc contribute to a variety of addiction-related emotional and motivational states (Wolf, 2010). Lacking apparent internal pacemaker mechanisms, NAc MSNs heavily rely on synaptic input to fire action potentials for their functional output (Wilson & Kawaguchi, 1996). Whereas synchronous excitatory synaptic input provides the major driving force to bring NAc MSNs to their functionally active state for action potential firing, inhibitory synaptic input makes NAc MSNs resistant to excitation (Gambrill & Barria, 2011; O'Donnell & Grace, 1995; Wilson & Kawaguchi, 1996; Wilson, 1986). Therefore, the inhibitory synaptic inputs to the NAc contribute to the overall output of NAc MSNs. Also, the inhibitory efficacy is determined by its reversal potential. Typically, the more hyperpolarized the reversal potential from the resting membrane potential, the more inhibitory control it can provide. Thus, using gramicidin-perforated patch, we first investigated the reversal potential of IPSCs in NAc MSNs. Before starting the planned experiments, we decided to validate our experimental system using animals of different ages

³ The work presented in Chapter 4 is from "Cocaine-induced adaptations in GABAergic synaptic transmission to the nucleus accumbens." M. Otaka, M. Ishikawa, B.R. Lee, Y.H. Huang, O.M. Schlüter, & Y. Dong. 2011. Poster presentation. Annual meeting of the Society for Neuroscience, Washington D.C. and from "Exposure to cocaine regulates inhibitory synaptic transmission in the nucleus accumbens." M. Otaka*, M. Ishikawa*, B.R. Lee, L. Liu, P.A. Neumann, R. Cui, Y.H. Huang, O.M. Schlüter, and Y. Dong. 2013. *J Neurosci*, 33(16), 6753-6758.

following the general properties of GABAergic synapses during development (Ben-Ari, Cherubini, Corradetti, & Gaiarsa, 1989). Our results showed that the reversal potential of IPSCs in NAc MSNs was substantially more depolarized in young rats (P6) than older rats (Fig. 11A–C), consistent with the previous reports. After the validation of our experimental system, we examined whether the chloride reversal potential for rats treated with i.p. cocaine injection is altered. Our results demonstrated that re-exposure to cocaine during 3 weeks of withdrawal from passive exposure to cocaine (challenge exposure, 15 mg/kg/day x 5 consecutive days) significantly shifted the reversal potential towards a more depolarized direction (Fig. 12). These results suggest that inhibitory synaptic transmission, together with excitatory synaptic transmission and the membrane excitability, are primary cellular targets for cocaine exposure to produce cellular and behavioral alterations. Balanced in a finely tuned excitatory/inhibitory (E/I) synaptic integration, NAc MSNs maintain a stable functional state. Therefore, disrupting this E/I synaptic balance can be one way that exposure to cocaine reshapes the basal functional state of NAc MSNs. Although cocaine-induced alterations at excitatory synapses have been extensively examined, still only little is known about whether inhibitory synaptic input to NAc MSNs is affected following exposure to cocaine. Using contingent cocaine procedures, experiments in this chapter examine the impact of cocaine exposure on IPSCs and their relative weight to excitatory synaptic input to NAc MSNs in rats.

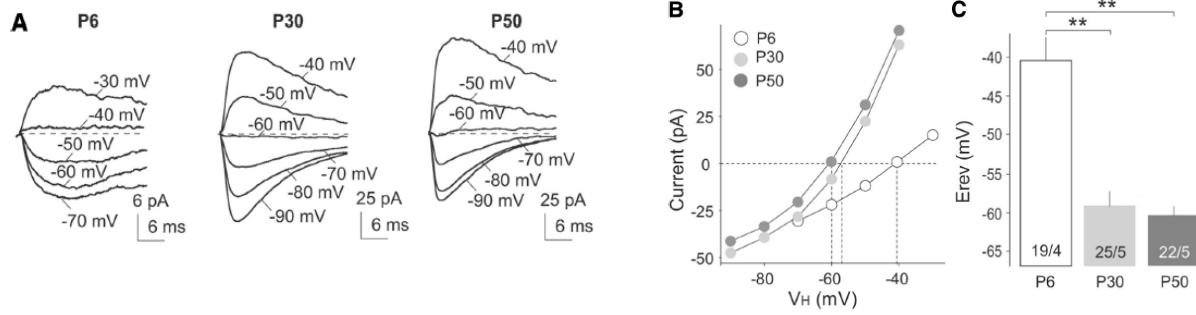


Figure 11. Young rats showed depolarized the reversal potential of IPSCs in NAc MSNs.

A. Example IPSCs elicited at different membrane potentials in NAc MSNs from rats at ages P6, P30, and P50. Recording made via perforated patch electrode.

B. The I-V curves of IPSCs in NAc MSNs from different ages of rats.

C. Summarized results showing different reversal potentials of IPSCs in NAc MSNs can be detected from different ages of rats.

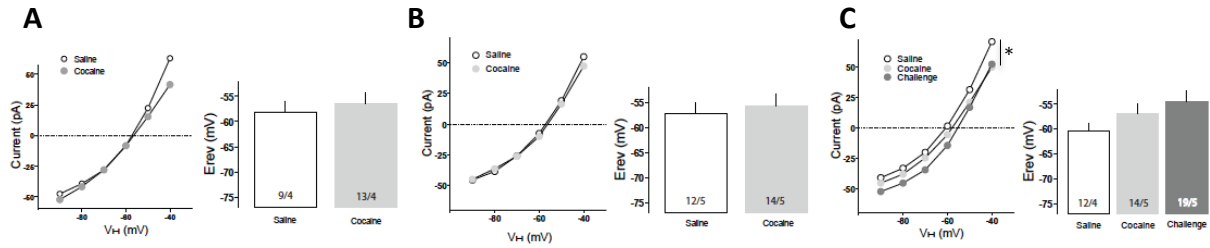


Figure 12. The Shift of chloride reversal potential after cocaine experience.

A. Acute i.p. injection of cocaine did not alter the chloride reversal potential.

B. 1 day withdrawal from 5 days repeated cocaine exposure by i.p. injections did not show the alteration in the chloride reversal potential.

C. Challenge injection of cocaine by i.p. injection after 21 days withdrawal from 5 days repeated cocaine i.p. injections significantly shift the chloride reversal potential to more depolarized direction.

4.2 MATERIALS AND METHODS

Subjects

The subjects were male Sprague Dawley rats from Simonsen (CA, USA) and Harlan Laboratories (MD, USA) and they were 30- to 40-days-old before surgery. Some rats were used at postnatal day 6 (P6) for controls. Rats were housed on a regular 12 h light/dark cycle (light on at 7:00 am) with food and water available ad libitum.

i.p. Cocaine Administration

Rats received repeated i.p. injections of cocaine (15 mg/kg in saline for 5 consecutive days) or saline in a novel environment, as described previously (T. E. Brown et al., 2011; Ishikawa et al., 2009; Mu et al., 2010). Rats were placed back to the home cage for withdrawal.

Intravenous Surgery and Self-Administration of Cocaine

Rats were anesthetized with a xylazine/ketamine mixture (5-10/50-100 mg/kg, i.p.). The catheter was inserted into the jugular vein passed subcutaneously to the mid-scapula and connected to a harness with quick connect luer (SAI Infusion Technologies, IL, USA). Rats were then single housed and given 5-7 days to recover before training session. After 5 days self-administration session (>10 infusion/2 hrs session/day at 0.75 mg/kg/infusion for cocaine), rats were placed into home cages for withdrawal. Catheters were flushed with sterile saline containing gentamicin (5 mg/ml) and heparin (10 U/ml) every 24 hrs during recovery, training/test session, and withdrawal. On withdrawal day 21, a subgroup of rats was placed back into the operant boxes for cocaine re-exposure. The re-exposure procedure was identical to the 2 hrs self-administration session.

Apparatus

The self-administration chambers, controlled by a Med Associates system, had two nosepoke holes located 6 cm above the grid floor, but only one hole (active hole) activated the infusion pump. The luer on the harness was connected to a liquid swivel with dual-luer spring tether and connected to the syringe of the infusion pump. Poking in the other hole (inactive hole) was also recorded. A nosepoke to the active hole resulted in a cocaine infusion, accompanied by the switch-on of a light as a conditioned stimulus (CS) and a background light. The CS stayed on for 6 s, and the background light for 20 s. During this 20 s, additional nosepokes were counted but did not result in cocaine infusion.

Preparation of acute brain slices

Rats were decapitated following deep isoflurane anesthesia. Coronal slices (250–300 μm) containing the NAc were prepared on a vibratome in 4°C cutting solution containing the following (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 , pH adjusted to 7.4 with HCl. Slices were incubated in 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, saturated with 95% O_2 /5% CO_2 at 37°C for 30 min and then allowed to recover for at least 30 min at room temperature before experimentation.

Electrophysiological recordings

All recordings were made from MSNs located in the NAcSh. During recordings, brain slices were superfused with aCSF that was heated to 31–33°C by passing the solution through a

feedback controlled in-line heater before entering the recording chamber. Recordings were made under visual guidance (40 or 60×, differential interference contrast optics) with electrodes (2–5MΩ) filled with the following (in mM): 140 CsCH₃O₃S (or CsCl for recording mIPSCs), 5 TEA-Cl, 0.4 Cs-EGTA, 20 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP, and 1 QX-314, pH 7.3. PTX (100 μM) (or APV, 50 μM) was included in the external perfusion aCSF to block GABA_AR (or NMDAR).

For mIPSC recordings, we used a high chloride cesium-based internal solution. The aCSF used in this experiment contained tetrodotoxin (TTX: 1 μM), APV (50 μM), and NBQX (5 μM). Cells were stabilized in TTX containing aCSF at least for 10 min. Because of the possibility of the blockade of action potential on synaptic properties, all mIPSC recordings were made within 30 min after TTX was applied to the brain slices (Winters et al., 2012).

For gramicidin-perforated patch, micropipettes were filled with an internal solution (100 mM KCl and 10 mM HEPES, pH 7.2) and the same solution, which contains 40 μg/μl gramicidin. IPSCs were recorded in the presence of APV (50 μM) and NBQX (2 μM) in standard.

To measure AMPAR/NMDAR ratio, we took the average of EPSCs at +50 mV in the absence and presence of APV (50 μM). Using the Clampfit, the average response in the presence of APV (i.e. AMPAR-only response) was subtracted from the average “total” response, which was recorded in the absence of APV (i.e. AMPAR+NMDAR response) and an average NMDAR EPSC was calculated. And then, the peak of the AMPAR EPSC was divided by the peak of the NMDAR EPSC (Kourrich et al., 2007; Thomas et al., 2001).

To measure AMPAR/GABAR ratio, we randomly placed a stimulation electrode within the NAc slice to elicit a compound current in NAc MSNs, containing both EPSC and IPSC (holding potential at -70 mV). We then perfused the slice with PTX (100 μM) to selectively inhibit IPSCs. Using the same way as AMPAR/NMDAR ratio, we subtracted the PTX-sensitive current

(i.e. AMPAR-only response) from the “total” response (i.e. AMPAR+GABAR response) and the GABAR only response was calculated. And then, the peaks of these responses were used for the ratio calculation.

To measure input-output efficacy, a combined excitatory and inhibitory input was evoked in each recorded NAc MSN by a stimulation electrode. The distance (100 μm), stimulation duration (0.25 ms), stimulation electrode size (2.5–3 $\text{M}\Omega$), and stimulation frequency (20 Hz) were fixed for all recordings. Micropipettes were filled with a potassium-based internal solution containing the following (in mM): 130 KMeSO_3 , 10 KCl, 0.4 EGTA, 10 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP, and 2 $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, pH 7.3. The stimulation intensity continuously increased by an increment of 2 μA . Presynaptic afferents were stimulated by a constant-current isolated stimulator (Digitimer), using a monopolar electrode (glass pipette filled with aCSF). Stimulus strength was adjusted so that the first EPSC/IPSC was between 100 and 500 pA. Trains of pulses were generated using Clampex software (Molecular Devices).

Series resistance was 9-20 $\text{M}\Omega$, uncompensated, and monitored continuously during recording. Cells with a change in series resistance beyond 15% were not accepted for data analysis. Synaptic currents were recorded with a MultiClamp 700B amplifier, filtered at 2.6–3 kHz, amplified five times, and then digitized at 20 kHz.

Drugs

TTX, APV, and NBQX were purchased from Tocris Bioscience (Bristol, UK). In addition, PTX, gramicidin, and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Data acquisition and statistics

All data were analyzed off-line. For mIPSC analysis, a template was made by averaging 200 hand-picked miniature events using pClamp10 software. Relative AMPAR/NMDAR ratio and AMPAR/GABAR ratio were calculated by normalizing the amplitude of the total control response to 1. All statistical results were expressed as mean \pm SEM. Two-tail t test or ANOVA was used for statistical comparisons as specified in the text.

4.3 RESULTS

The following study focused on MSNs within the ventrolateral NAcSh, a brain site that has been implicated in emotional and motivational responses (Kelley, 2004). To examine potential cocaine-induced adaptations at inhibitory synapses of NAc neurons, we used a 5 days cocaine self-administration procedure (Mu et al., 2010), in which rats nose-poked for intravenous injection of cocaine (Fig. 13A). After 1 day or 21 days withdrawal, rats were either killed or placed back to the operant boxes for another 2 hrs session (i.e., re-exposure) and then killed immediately for slice electrophysiology.

We first recorded mIPSCs from NAc MSNs. Our results show that the amplitude of mIPSCs was decreased after 21 days withdrawal from cocaine self-administration, and this decrease was not affected by cocaine re-exposure ($F_{(2,63)}=4.230$, $p<0.05$, one-way ANOVA; $p<0.05$, saline vs cocaine and saline vs re-exposure, $p=1.00$, cocaine vs re-exposure, Bonferroni post-tests; Fig. 13B,C). Furthermore, the frequency of mIPSCs was not affected after cocaine withdrawal but was significantly increased upon cocaine re-exposure ($F_{(2,63)}=3.719$, $p<0.05$, one-way ANOVA; $p=1.00$, saline vs cocaine; $p<0.05$, saline vs re-exposure; $p=0.132$, cocaine vs re-

exposure, Bonferroni post-tests; Fig. 13B,D). In general, alterations in the amplitude and frequency of miniature events reflect postsynaptic and presynaptic changes, respectively. Thus, these results suggest a decrease in postsynaptic responsiveness of GABAergic synapses in NAc MSNs after cocaine withdrawal and this adaptive change was not affected upon cocaine re-exposure, whereas presynaptic release was not altered after cocaine withdrawal but enhanced upon re-exposure to cocaine. Apparently, these presynaptic and postsynaptic changes functionally oppose to each other.

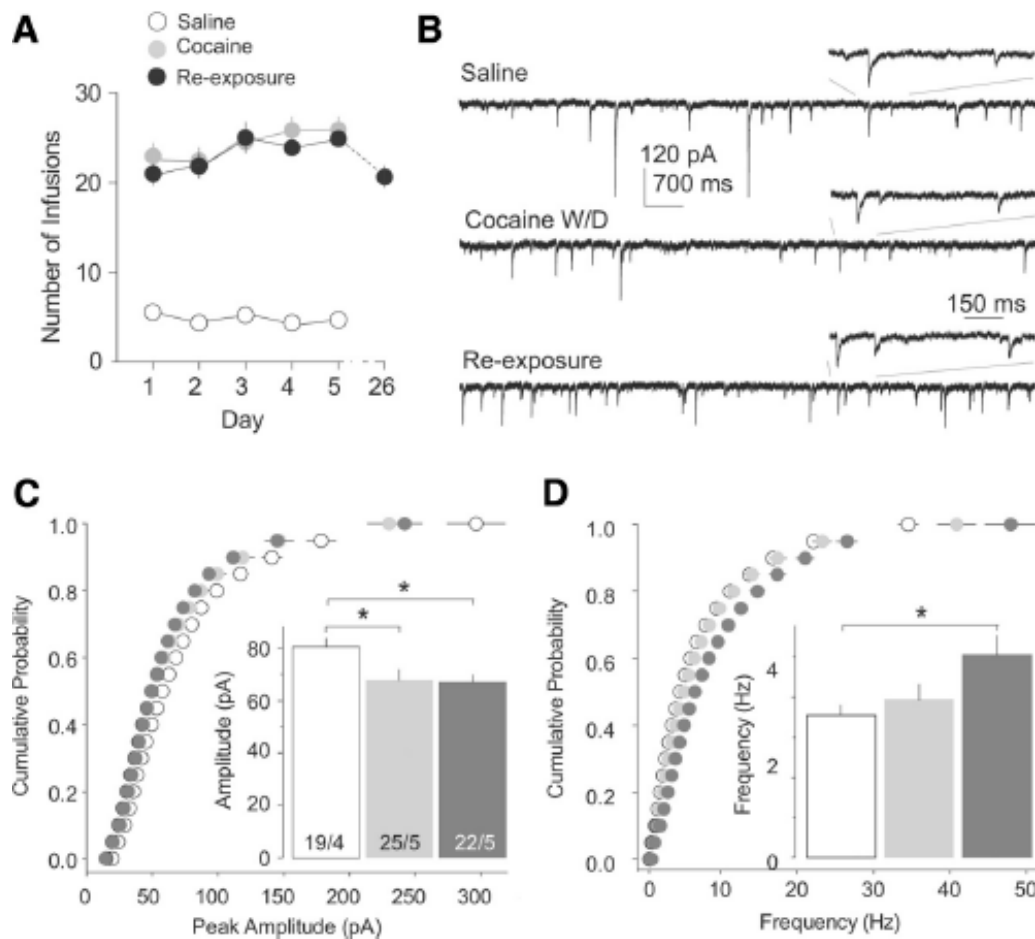


Figure 13. Regulation of mIPSCs in NAc MSNs after cocaine self-administration.

A. Summarized results showing a 5 d cocaine self-administration procedure in which rats established stable nose-pokes for cocaine infusions. On withdrawal day 21, a subgroup of rats was placed back into the operant boxes for an additional session of cocaine self-administration (re-exposure). Data include all rats in the current study.

B. Example mIPSCs in NAc MSNs from rats with saline self-administration and 21 d withdrawal (upper); rats with cocaine self-administration and 21 d withdrawal (middle); and rats with cocaine self-administration, 21 d withdrawal, and cocaine re-exposure (lower). Insets showing mIPSCs with slower time scale.

C. Summarized results showing that the peak amplitude of mIPSCs was decreased after cocaine withdrawal and this decrease was not affected by cocaine re-exposure.

D. Summarized results showing that the frequency of mIPSCs was not affected after cocaine withdrawal but was increased upon re-exposure to cocaine after withdrawal. * $p < 0.05$.

Because the inhibitory efficacy is determined by its reversal potential, we next measured the reversal potential of IPSCs in NAc MSNs from rats trained with cocaine self-administration, using gramicidin perforated patch technique (Fig. 14A). Our results show that the reversal potential of IPSCs in NAc MSNs was not affected after 21 days withdrawal from cocaine but became significantly hyperpolarized upon cocaine re-exposure ($F_{(2,63)}=3.605$, $p<0.05$, one-way ANOVA; $p<0.05$, cocaine vs re-exposure, $p=0.21$, saline vs re-exposure; Bonferroni post-tests; Fig. 14B,C). This cocaine-induced change may provide stronger inhibitory control from GABAergic synapses to dampen the excitation of NAc MSNs after re-exposure to cocaine. Because NAc MSNs do not possess intrinsic pace-making mechanisms, the functional output is critically dependent on the integration of excitatory and inhibitory synaptic inputs.

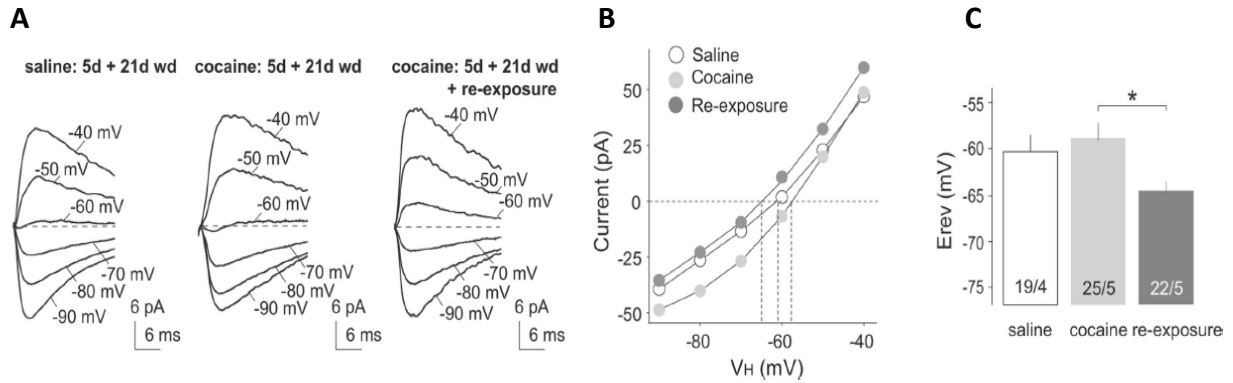


Figure 14. Re-exposure to cocaine after withdrawal hyperpolarizes the reversal potential of IPSCs in NAc MSNs.

A. Example IPSCs elicited at different membrane potentials from rats with saline self-administration and 21 d withdrawal; rats with cocaine self-administration and 21 d withdrawal; and rats with cocaine self-administration, 21 d withdrawal, and re-exposure to cocaine on withdrawal day 21.

B. The I–V curves of IPSCs in NAc MSNs from rats with different drug administration procedures.

C. Summarized results showing that the reversal potential of IPSCs in NAc MSNs was not affected after withdrawal from cocaine self-administration but became hyperpolarized upon re-exposure to cocaine after withdrawal. * $p < 0.05$; ** $p < 0.01$.

We next examined whether the balance between E/I synaptic inputs was affected by cocaine self-administration. Consistent with cocaine-induced alterations at excitatory synapses in NAc MSNs following passive procedures of cocaine injections (Kourrich et al., 2007), we observed an increase in the ratio of AMPAR- and NMDAR-mediated EPSCs in NAc MSNs after withdrawal from cocaine self-administration, and this AMPAR/NMDAR ratio returned to the basal level upon cocaine re-exposure ($F_{(2,12)}=16.51$, $p<0.001$, one-way ANOVA; $p<0.01$, saline vs cocaine and cocaine vs re-exposure; $p=1.00$, saline vs re-exposure, Bonferroni posttests; Fig. 15A,B). This result, together with previous biochemical studies measuring surface AMPAR subunits (Conrad et al., 2008; Wolf, 2010), suggests that the excitatory synaptic strength was increased after withdrawal but returned to the basal level upon cocaine re-exposure following cocaine self-administration. To examine the effect of cocaine on the E/I in NAc MSNs, we measured the relative weight of excitatory and inhibitory synaptic inputs. Briefly, we randomly placed a stimulation electrode within the NAc slice to elicit a compound current in NAc MSNs, containing both EPSC and IPSC (Fig. 15C). We then perfused the slice with PTX (100 μ M) to selectively inhibited IPSCs (Fig. 15C). By subtracting the PTX-sensitive current from the total current, we obtained EPSCs (Fig. 15C), which was sensitive to AMPAR-selective antagonist NBQX (data not shown). The E/I was thus operationally defined as the ratio of the peak amplitude of EPSCs divided by the peak amplitude of IPSCs. The E/I was significantly decreased after 1 day withdrawal from cocaine self-administration ($t_{(8)}=4.65$, $p<0.01$, saline vs cocaine), but increased after 21 days withdrawal, and returned to the basal level upon cocaine re-exposure after withdrawal ($F_{(2,12)}=49.25$, $p<0.0001$, one-way ANOVA; $p<0.001$, saline vs cocaine and cocaine vs re-exposure; $p=0.08$, saline vs re-exposure, Bonferroni post-tests; Fig. 15C,D). Thus, the net change of the E/I in NAc MSNs is dynamic following cocaine self-administration. Our present findings

together with previous results demonstrate dynamic alterations of the membrane excitability and E/I in the NA during cocaine withdrawal and upon re-exposure to cocaine after withdrawal.

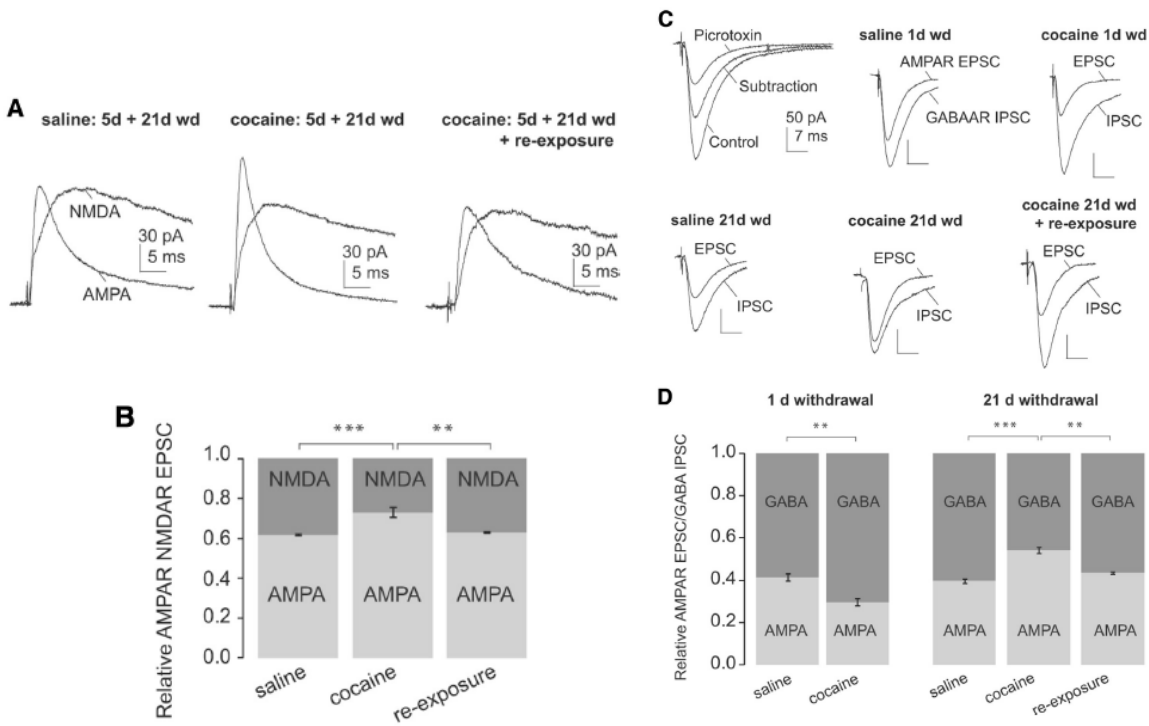


Figure 15. The AMPAR/NMDAR and AMPAR/GABA ratios in NAc MSNs are dynamically regulated after cocaine self-administration.

A. Example AMPAR and NMDAR EPSCs in NAc MSNs from rats with saline self-administration and 21 d withdrawal; rats with cocaine self-administration and 21 d withdrawal; and rats with cocaine self-administration, 21 d withdrawal, and re-exposure to cocaine after withdrawal.

B. Summarized results showing that the AMPAR/NMDAR ratio was increased after long-term withdrawal from cocaine self-administration, but this withdrawal effect was abolished upon cocaine re-exposure.

C. Example traces showing IPSCs and EPSCs in the same NAc MSNs from rats with 1 or 21 d withdrawal from saline or cocaine self-administration, or with a re-exposure to cocaine after 21 d withdrawal. The upper left traces show pharmacological isolation of IPSCs and EPSCs. Briefly, total (control) synaptic currents were recorded from NAc MSNs containing both IPSCs and EPSCs. Perfusion of picrotoxin selectively inhibited IPSCs. EPSCs were obtained by subtracting picrotoxin-sensitive currents from the total currents.

D. Summarized results showing that the AMPAR/GABA ratio was significantly decreased after 1 d withdrawal from cocaine self-administration but increased after 21 d withdrawal, and this change was abolished upon re-exposure to cocaine. ** $p < 0.01$; *** $p < 0.001$.

In an attempt to determine the integrated effect of these alterations on the overall functional output of NAc MSNs, we examined the input-output efficacy by measuring synaptically driven action potential firing in NAc MSNs (Hopf, Cascini, Gordon, Diamond, & Bonci, 2003). Specifically, a combined excitatory and inhibitory input was evoked in each recorded NAc MSN by a stimulation electrode. Net depolarizing synaptic potentials (a combination of EPSPs and IPSPs) were observed upon these stimulations, and action potentials were evoked when the synaptically driven depolarization drove the membrane potential beyond the threshold of action potentials (Fig. 16A) (Hopf et al., 2003). After 1 day withdrawal from cocaine self-administration, the input-output efficacy was significantly decreased ($F_{(49, 637)}=1.80$, $p<0.001$; Fig. 16B). The input-output efficacy remained low after 21 days withdrawal but was significantly increased after re-exposure to cocaine after 21 days withdrawal ($F_{(98, 1274)}=3.44$, $p<0.001$, saline vs cocaine; $p<0.01$ cocaine vs re-exposure; two-factor ANOVA with Bonferroni posttests; Fig. 16C).

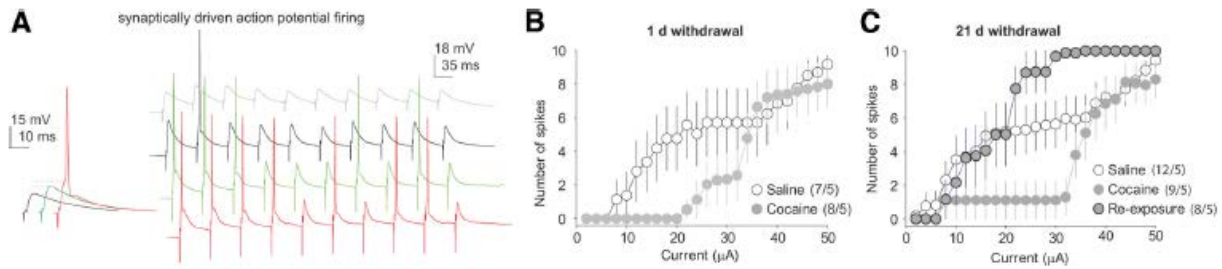


Figure 16. Synaptically driven action potential firing is dynamically regulated after cocaine self-administration.

A. Example traces of synaptically driven action potential firing in NAc MSN. Left, Voltage traces from an example NAc MSN show that the synaptically driven depolarization of membrane potential increased in response to increased intensity of stimulations (12, 14, and 16 μA). Note that the stimulation artifacts preceded the synaptically driven membrane depolarization and action potentials, suggesting they were synaptically evoked events. Right, Voltage traces from an example NAc MSN show synaptically driven action potential firings (stimulation intensity, 12, 18, 24, and 32 μA).

B. Summarized results showing that after 1 d withdrawal from cocaine self-administration the input-output efficacy was significantly decreased in NAc MSNs. Note that the decrease became minimal when the synaptic stimulations were strong (right part of the curve).

C. Summarized results showing that after 21 d withdrawal from cocaine self-administration, the input-output efficacy of NAc MSNs remained low upon relatively weak synaptic activations (left part of the curve), and re-exposure to cocaine instantly increased the input–output efficacy beyond the basal (saline control) level during relatively strong synaptic activations (right part of the curve).

4.4 DISCUSSION

By measuring different aspects of GABAergic synaptic input, our study is among the first showing dynamic changes at inhibitory synapses in the NAc following cocaine self-administration. These results may help generate an overall picture of the functional alterations of NAc MSNs during drug withdrawal and relapse.

Withdrawal

After 1 day withdrawal from cocaine self-administration, the E/I ratio was significantly decreased (Fig. 15D), which can be partially mediated by the reduced excitatory synaptic strength (Wolf, 2010). These synaptic adaptations together with cocaine-induced decreases in the membrane excitability (Mu et al., 2010) may contribute to the decreased input-output efficacy (Fig. 16B). Thus, the overall functional output of NAc MSNs is likely to be decreased after short-term cocaine withdrawal. After 21 days withdrawal, the input-output efficacy remained low (Fig. 16C). At this time point, the postsynaptic responsiveness of GABAergic synapses in NAc MSNs appears to be reduced (Fig. 13C), whereas the excitatory synaptic input appears to be enhanced both presynaptically (Suska et al., 2013) and postsynaptically (Conrad et al., 2008; Wolf, 2010) (Fig. 15). These synaptic adaptations may together contribute to the increased E/I ratio observed in NAc MSNs at this withdrawal time point (Fig. 15D), and these synaptic adaptations function to promote the excitation of NAc MSNs. On the other hand, the intrinsic membrane excitability of NAc MSNs returns close to the saline control level, but a minimal decrease still remains (Mu et al., 2010). A decreased input-output efficacy (Fig. 16C) thus suggests that the cocaine-induced membrane adaptation, although small, predominates and decreases the overall functional output

of NAc MSNs. It is important to note that the above cellular changes were detected in the condition that E/I inputs are experimentally normalized. That is, upon the same synaptic input, NAc MSNs would respond less actively after cocaine withdrawal. However, excitation of NAc MSNs in vivo relies on synaptic inputs. As such, even if NAc MSNs are set to be less responsive, their excitation state can still be high in behaving animals if there is increased excitatory input.

Re-exposure

Upon re-exposure to cocaine, the presynaptic potentiation (Fig. 13D) combined with the hyperpolarized reversal potential may effectively enhance inhibitory synaptic input to NAc MSNs. At the same time, the withdrawal-induced up-regulation of excitatory synaptic strength may transiently decrease to the basal level (i.e., saline control). These synaptic changes integrate, potentially leading to the return of E/I ratio to the basal (control) level (Fig. 15D). On the other hand, the intrinsic membrane excitability of NAc MSNs is substantially increased upon re-exposure to cocaine (Mu et al., 2010). This membrane change may predominate in synapse-membrane integration, resulting in an increase in the input-output efficacy of NAc MSNs, especially upon strong synaptic input (Fig. 16C).

5.0 GENERAL DISCUSSION

Summary and Interpretation of Findings: VTA-to-NAc GABAergic projection

It has long been known that physiological as well as pathophysiological emotional and motivational states are critically regulated by the mesolimbic DA system, mainly comprising the synaptic projection from the VTA to the NAc. Recent discoveries have revealed a complex nature of the VTA-to-NAc projection. In addition to the neuromodulator DA, activation of the VTA-to-NAc projection also releases a rich repertoire of neurotransmitters, in particular, glutamate and GABA, which mediate fast excitatory and inhibitory synaptic transmissions, respectively. In contrast to DA and other neuromodulators, these fast synaptic transmissions can instantly change the membrane potential of NAc neurons upon VTA activation, exerting highly timing-contingent regulation of the mesolimbic output.

- *Exposure to cocaine regulates fast synaptic transmissions from the VTA to the NAc*

Results from Chapter 2 characterize basic physiological properties of the VTA-to-NAcCo projection and cocaine-induced adaptations in this projection. Our results show that VTA-to-NAc fast excitatory transmission is not altered either pre- or postsynaptically after short-term withdrawal from repeated non-contingent cocaine exposure. Such a ‘drug-resistant’ feature at VTA-to-NAcCo excitatory synapses may not apply to other excitatory inputs because a study indicates the overall increased AMPAR/NMDAR ratio in the NAcCo, due to reduction of D-serine binding and thus hypofunction of NMDARs (Curcio et al., 2013). Nonetheless, our results indicate that VTA-to-NAc fast glutamatergic synaptic transmission is relatively weak compared to randomly sampled synapses, and the postsynaptic properties are different between

VTA-to-NAcCo and randomly sampled excitatory synapses (Fig. 3) Also, the postsynaptic properties are not affected by cocaine exposure. These facts suggest that VTA-to-NAcCo fast glutamatergic transmission is unlikely to be the primary contributor to the overall excitatory inputs onto the NAcCo.

On the other hand, VTA-to-NAcCo fast inhibitory synaptic transmission may play a critical role in inhibiting NAcCo MSNs. Our results show that VTA-to-NAcCo GABAergic projection is inhibited via presynaptic mechanisms after short-term withdrawal from repeated cocaine exposure (Fig. 5). This disinhibition may cause the shift of NAcCo MSNs to more excitable conditions. The increased activation of NAcCo MSNs may enhance the drug-seeking behavior.

In addition to our data, alterations in VTA GABAergic neurons after cocaine exposure have been reported. A previous study shows that cocaine inhibits the excitability of VTA GABAergic neurons, and this effect disinhibits DAergic neurons in the VTA (Steffensen et al., 2008). Another group indicates the potentiation of GABAergic input from NAc D1-expressing MSNs to the VTA GABAergic neurons at this withdrawal time, leading to the disinhibition in VTA DAergic neurons (Bocklisch et al., 2013). The activity of NAc D1-expressing GABAergic MSNs may affect the long-range GABAergic projection, resulting in decreased presynaptic release within the NAcCo after short-term withdrawal. It is important to note that GABAergic neurons appear to make a complicated disinhibition loop in the VTA (Omelchenko & Sesack, 2009). When the local VTA GABAergic neurons and the long-range GABAergic neurons receive the NAc-mediated GABAergic control, it is possible that the local GABA neurons decreased activity and potentiate the activity of the long-range GABAergic neurons, resulting the enhanced inhibitory influence in the NAc. However, our results exhibit the inhibition of the long-range GABAergic projection. This indicates the importance to study

whether the two types of GABAergic neurons receive NAc-mediated inhibition in same degree or not. Also, further investigation, as such how both types of VTA GABAergic neurons are interacted and affected by cocaine exposure, is necessary to confirm this speculation.

While VTA-to-NAcCo GABAergic transmission is inhibited after short-term withdrawal, it returns to the basal level after long-term withdrawal. At the same time, VTA-to-NAcCo excitatory transmission is presynaptically enhanced. Previous studies also suggest the up-regulation of AMPARs in the NAcCo at this withdrawal point (Wolf & Tseng, 2012). Our result showing presynaptic enhancement in the VTA-to-NAcCo excitatory synapses may be accompanied with the enhanced postsynaptic AMPAR function at this withdrawal point. These results suggest an overall enhancement of excitatory synapses in the NAcCo, both pre and postsynaptically. Even combining with the restored inhibitory tone, this enhanced excitatory synapses may cause the overall balance shifted to excitatory.

These dynamic alterations of VTA-to-NAc excitatory and inhibitory transmissions at different withdrawal times shift the excitatory and inhibitory balance. Both short- and long-term withdrawals seem to cause the shift to the excitatory direction. This shift makes the NAcCo more excitable and may have a link to the drug-seeking behavior.

- *Dopamine triggers heterosynaptic plasticity and the effect from cocaine exposure*

Alterations in VTA-to-NAcCo inhibitory transmission after cocaine exposure make us consider the effect on VTA GABAergic projection from the neighboring DAergic projection because the VTA GABAergic projection is activated by the same emotional/motivational stimuli (Carr & Sesack, 2000; Steffensen et al., 1998). These anatomical and physiological patterns suggest that these two projections may have a unique interaction. Also, it has been

reported that cocaine exposure affect inhibitory long-term plasticity at the VTA GABAergic synapses. Cocaine-induced eCB-mediated iLTD at VTA GABAergic synapses on VTA DAergic neuron has been observed after repeated cocaine exposure (Woodin & Maffei, 2011), which disinhibits VTA DAergic neurons. In addition, there is a finding that indicates the impairment of iLTP 24 hours after cocaine exposure on VTA DAergic neurons (Niehaus, Murali, & Kauer, 2010). Thus, we investigated iLTD at the VTA-to-NAcCo GABAergic projection. Our work demonstrates that the induction of iLTD requires DAergic signaling (Fig. 7). The following observations confirmed that the iLTD is triggered by the parallel DAergic signaling (Fig. 8&9)

Our data exhibit that this DA-triggered heterosynaptic iLTD is abolished after long-term withdrawal from non-contingent cocaine experience; the disruption of this heterosynaptic iLTD may result in compromised flexibility of NAcCo to process incentive stimuli after cocaine withdrawal. Results from previous chapter show the presynaptic activity of VTA GABAergic neurons is restored after long-term withdrawal, which is accompanied by the impairment of DA-triggered iLTD.

On the other hand, during short-term withdrawal, we did not detect the alteration in this iLTD. This difference between two different withdrawal points may be due to the activity of VTA DAergic neurons after repeated cocaine exposure and the subsequent withdrawal. Some studies indicate that activity of VTA DAergic neurons after drug exposure is transient. The firing is decreased during drugs are in the system, whereas the firing rate and bursting of VTA DAergic neurons after short-term withdrawal are increased. Also, this increased firing disappears after about 2 weeks (Wolf & Tseng, 2012). Because a form of iLTD found in our study requires VTA DA signaling, this transient activity of VTA DAergic neuron may play a

role in the impaired iLTD during long-term withdrawal. Further experiment using the animals after acute cocaine exposure and testing VTA DAergic activity after 3 weeks withdrawal, may help to explain the difference we observed.

Overall, these above results suggest that action-potential-dependent DA release triggers very different cellular consequences. Activation of the VTA-to-NAcCo projections is essential for emotional and motivational responses. This DA-triggered iLTD allows a flexible output of NAcCo neurons, whereas disruption of this iLTD may contribute to the rigid emotional and motivational state observed in addicts during cocaine withdrawal.

- *Exposure to cocaine regulates inhibitory synaptic transmission in the NAc*

In addition to the VTA-to-NAc projection, NAc MSNs receive multiple other excitatory and inhibitory synaptic inputs. After characterizing cocaine-induced alterations in VTA-to-NAc inhibitory synaptic transmission, I subsequently determined whether the overall inhibitory input in the NAc MSNs is altered by cocaine experience. Because there is no internal pace-making mechanism, NAc MSNs are highly dependent on synaptic inputs to fire action potentials for their functional output (Wilson & Kawaguchi, 1996). A relatively stable excitatory and inhibitory synaptic balance is necessary for maintaining a stable functional output. We have known that the excitatory synaptic transmission is altered after cocaine exposure. However, alteration in the inhibitory synaptic transmission in the NAc after cocaine experience has not been well documented. This lack of knowledge limits our understanding how cocaine experience affects the excitatory and inhibitory interaction and balance. Results from Chapter 4, combined with the results from other chapters in this dissertation and the context of current literature, provide a relatively thorough picture of the functional state of

NAc MSNs following cocaine exposure by factoring in cocaine-induced dynamical alterations in synaptic and membrane properties.

Cocaine-induced adaptations in the NAc MSNs

- *Non-contingent: Short-term withdrawal*

According to our and available data, cocaine exposure may affect the NAcSh and the NAcCo differently. During short-term withdrawal, surface AMPARs remain unchanged (Boudreau et al., 2007; Boudreau & Wolf, 2005), and AMPAR/NMDAR ratio is decreased (Kourrich et al., 2007) in NAcSh MSNs. This decrease may be partially mediated by synaptic insertion of new NMDARs (Y. H. Huang et al., 2009). On the other hand, in NAcCo MSNs, one study suggests that the AMPAR-mediated current does not show significant changes (Kourrich & Thomas, 2009), and a report indicates increased AMPAR/NMDAR ratio (Curcio et al., 2013). Alterations in membrane excitability seems more dynamic between two subregions; the membrane excitability in the NAcSh is decreased, whereas NAcCo MSNs exhibit increased membrane excitability (Kourrich & Thomas, 2009).

The underlying mechanisms of this different firing between NAcSh and NAcCo neurons are still unknown, but alteration in NMDAR after cocaine exposure within two subregions may explain this situation. Previous work from our laboratory demonstrated the existence of synapse-membrane homeostatic regulation, called homeostatic synapse driven membrane plasticity (hSMP), as one of the important mechanisms for maintaining functional output of NAc MSNs (Ishikawa et al., 2009). Specifically, NAc MSNs adjust their membrane excitability to compensate for the alteration in excitatory synaptic input. Additional results suggest that the increase in GluN2B-containing NMDARs in NAcSh neurons after short-term

withdrawal may produce a false signal of increased excitatory synaptic strength, even though synaptic AMPAR is not up-regulated, and this false homeostatic signal may trigger hSMP to decrease the membrane excitability (Y. H. Huang, Schlüter, & Dong, 2011). On the other hand, a study suggests the hypofunction of NMDARs in the NAcCo at this time point (Curcio et al., 2013). If this hypofunction exists after animals age and considered as decreased excitatory synaptic strength, it leads the increased membrane excitability in the NAcCo.

It is important to note that hSMP is one of the mechanisms to contribute to the alteration in the membrane excitability and the involvement of other mechanisms needs to be considered. Further investigations in ionic conductances are required to understand the overall picture (Section *Future direction: Impact of membrane excitability*).

Repeated cocaine exposure also affects inhibitory synapse. A previous report demonstrates that repeated cocaine exposure increases the spontaneous IPSC frequency in NAcSh MSNs with lack of alterations in the amplitude (Kennedy et al., 2013). On the other hand, we observed the decreased GABA release from the presynaptic site (Fig. 5), which indicate the decreased inhibitory tone to the NAcCo from the VTA. On the other hand, there is no evidence indicating the postsynaptic alterations in GABAergic synapses at this withdrawal point.

Overall, based on available data, the overall activity of the NAcSh seems to be decreased, whereas that of the NAcCo seems to be increased. These opposite overall activities may play a role in addiction-related behaviors.

- *Non-contingent: Long-term withdrawal*

Long-term withdrawal from cocaine exposure also appears to lead to different effects in the NAcSh and the NAcCo. Evidence shows an increase in AMPARs in both the NAcSh and

the NAcCo (Boudreau et al., 2007; Boudreau & Wolf, 2005; Y. H. Huang, Schlüter, et al., 2011; Kourrich et al., 2007). Alterations in the membrane excitability after long-term withdrawal have been reported: it stays decreased in NAcSh neurons, whereas it returns to the basal level in NAcCo neurons (Ishikawa et al., 2009; Kourrich & Thomas, 2009; Mu et al., 2010).

These differences in the NAcSh and the NAcCo after long-term withdrawal appear to affect the membrane excitability through hSMP. In the NAcSh, the increased NMDAR signal contributes to the decreased membrane excitability via hSMP, similar to the situation in the short-term withdrawal. On the other hand, in the NAcCo, the excitatory synaptic inputs seem to be increased. This significant increase may lead to the decrease in membrane excitability. We do not know whether the hypofunction of NMDARs reported in the short-term withdrawal still remain, but if it does, this could be a reason why the membrane excitability is higher than that of the NAcSh.

Alterations in inhibitory transmission are also observed at this withdrawal point. At VTA-to-NAcCo inhibitory synapses, the decreased GABA release seems to be recovered (Fig. 5), and DA-triggered iLTD can no longer be induced (Fig. 10). These results suggest a partial restoration of inhibitory synaptic transmission within the NAcCo from cocaine-induced alterations observed during short-term withdrawal. Also, a report shows the increase of basal GABA level in the NAc after long-term withdrawal from non-contingent repeated cocaine administration (Xi et al., 2003). Data indicating alterations in postsynaptic GABAR function after cocaine exposure have discrepancy: Some indicate the decreased function, but not all (Goeders, 1991; Peris, 1996; Suzuki et al., 2000; Xi et al., 2003).

Overall, by considering the alterations in the excitatory/inhibitory synapses and the

membrane excitability, the overall functional output of NAcCo MSNs may be more excitable state because of further enhancement of AMPAR-mediated inputs. This increase in the NAcCo activity may influence the drug-seeking behavior. On the other hand, it is difficult to speculate the overall activity of NAcSh MSNs because data to indicate the alteration in inhibitory synapses in the NAcSh are not sufficient during long-term withdrawal. However, the decreased membrane excitability may lead to decreased overall functional output in the NAcSh.

- *Non-contingent: Cocaine challenge after long-term withdrawal*

Compared to other conditions discussed above, much less information is available about re-exposure to cocaine after long-term withdrawal. At this point, I focus on cocaine-induced adaptations in the NAcSh. The AMPAR/NMDAR ratio is decreased after a cocaine challenge injection (Kourrich et al., 2007) and the membrane excitability is back to the basal level upon the challenge i.p. injection of cocaine after long-term withdrawal in the NAcSh (Y. H. Huang, Schlüter, et al., 2011; Mu et al., 2010). The recording times after challenge injection are different in above two studies; Kourrich et al. performed the electrophysiological recording 24 hours after challenge injection, whereas a study from Mu et al. prepared for the membrane excitability recordings about 1 hour after challenge injection. In addition, other studies suggest no change or increased AMPARs 30 minutes after challenge injection (Ferrario et al., 2010; Schierberl et al., 2011; Wolf & Tseng, 2012).

To my knowledge, no other published results are available about the effect of cocaine challenge on inhibitory transmission within the NAc MSNs except our result (Fig. 12) showing a shift in the reversal potential. Because the chloride reversal potential becomes more depolarized, there is less inhibitory control after cocaine challenge injection. These results

suggest that MSNs in the NAcSh may become easier to be excited after cocaine challenge injection.

- *Contingent: Short-term withdrawal*

Compared to non-contingent exposure cellular alterations at excitatory synapses, cocaine-induced adaptations after contingent exposure to cocaine seem to be more persistent in both the VTA and NAc (Wolf & Tseng, 2012). These persistent changes may play critical roles in the development of addiction-related behaviors (van Huijstee & Mansvelder, 2015). During short-term withdrawal after repeated contingent exposure to cocaine, the amplitude of mEPSC is decreased in NAcSh MSNs (Ortinski, Vassoler, Carlson, & Pierce, 2012), and this result is consistent with the decreased postsynaptic AMPAR expression (Conrad et al., 2008; Schumann & Yaka, 2009). Data from our laboratory indicate that synaptic NMDARs in the NAcSh are up-regulated in certain excitatory projections, which may further contribute to the decreased AMPAR/NMDAR ratio (Y. H. Huang, Schlüter, et al., 2011; B. R. Lee et al., 2013; Ma et al., 2014). At this time, the membrane excitability is decreased in NAcSh MSNs (Mu et al., 2010).

Unfortunately, data related to the inhibitory transmission are highly limited, and it is difficult to determine the “direction” of cocaine-induced alteration. Our data show that the AMPAR/GABAR ratio is significantly decreased (Fig. 15), suggesting either no change, an increase, or decrease in smaller degree (compared to the change in the AMPARs) of inhibitory transmission at this point. Overall, these changes may decrease the functional output of NAcSh MSNs. Also, our current results demonstrate that the input-output efficacy of NAcSh MSNs is dramatically suppressed (Fig. 16).

- *Contingent: Long-term withdrawal*

Many studies focusing on long-term withdrawal from cocaine exposure target post-withdrawal behaviors, such as cocaine craving and relapse. The involvement of glutamate system is extensively investigated (Wolf & Tseng, 2012). After long-term withdrawal from contingent exposure to cocaine, the excitatory synaptic strength is increased both pre- (Suska et al., 2013) and postsynaptically (Conrad et al., 2008; Wolf, 2010), and the AMPAR/NMDAR ratio is increased (Ortinski et al., 2012). Many electrophysiological studies, including our present data (Fig. 15), have been limited to the NAcSh, but available evidence indicates AMPAR up-regulation in the NAcCo as well (Wolf, 2010). Furthermore, it has been known that the up-regulated AMPARs could be different depending on the self-administration procedures. In a simplified view, the up-regulated AMPARs in limited access cocaine self-administration seem to be CI-AMPARs, whereas the up-regulated AMPARs in extended access (plus prolonged withdrawal) seem to be CP-AMPARs. However, data from our laboratory indicate that prolonged withdrawal from the limited access procedure may result in up-regulation of CP-AMPARs at certain NAc excitatory synapses (B. R. Lee et al., 2013; Ma et al., 2014). Our data in this dissertation, which show the increased AMPAR/NMDAR ratio after 21 days withdrawal from a limited access cocaine regimen (Fig. 15), match the current findings. Also, the up-regulated AMPARs in our experiments can be either/both types of AMPARs.

Taken together, the overall synaptic strength seems to be dramatically enhanced after long-term withdrawal from cocaine. However, results from our laboratory show that the membrane excitability at this point is almost back to the basal level, albeit a slight decrease being detected

(Mu et al., 2010). Why does hSMP lose its coordinative control over the excitatory synaptic strength and membrane excitability of NAc MSNs at this stage? A possible reason is the CP-AMPA up-regulation. Although the up-regulated AMPARs at this withdrawal point seem to be predominantly CI-AMPARs, CP-AMPARs may start to be recruited (B. R. Lee et al., 2013; Ma et al., 2014). In addition, although both NMDAR and CP-AMPAs conduct calcium ions, previous studies indicate that calcium signaling from NMDARs and CP-AMPARs are different (Y. H. Huang, Schlüter, et al., 2011). If CP-AMPARs and NMDARs play opposite roles in hSMP, the increase in CP-AMPARs may shift the membrane excitability back to the basal level.

Again, it is important to remember that hSMP is not a complete mechanism to explain the membrane adaptation after cocaine exposure. The alterations in other ionic conductances may be dynamic at this withdrawal point.

On the other hand, our results exhibited that the AMPAR/GABAR ratio is increased (Fig. 15), and the postsynaptic responsiveness of GABAergic synapses is decreased (Fig. 13). In addition, there is a report showing that the overall GABA level is decreased (Miguéns et al., 2011). These findings suggest a decreased inhibitory tone in NAcSh MSNs.

Overall, our data show that input-output efficacy is decreased at this withdrawal point (Fig.16). This indicates that the slight decrease in the membrane excitability may be dominant over the synaptic adaptations.

- *Contingent: Re-exposure after long-term withdrawal*

The effects of contingent cocaine re-exposure on NAc MSNs have not been extensively explored. Our previous study detects significant enhancement of membrane excitability (Mu

et al., 2010) after re-exposure to cocaine. Our present results show presynaptic potentiation of inhibitory synapses (Fig. 13) and hyperpolarized chloride reversal potential (Fig. 14). These data indicate that inhibitory control in the NAcSh may be stronger after re-exposure to cocaine. In addition, both the AMPAR/NMDAR ratio and AMPAR/GABAR ratio fall back to the basal level (Fig. 15). However, we do not know how long this effect lasts.

Combining available data, the enhanced input-output efficacy after re-exposure to cocaine (Fig. 16) may be induced predominantly by the increased membrane excitability.

GABAergic synapses in the NAc

NAc MSNs receive fast inhibitory synaptic inputs from multiple brain regions, including the VP, VTA, and local connections with other MSNs and interneurons (Faure et al., 2010). Compared with cocaine-induced adaptations at excitatory synapses and in the membrane excitability, much less is understood about how cocaine exposure affects inhibitory synaptic transmission in the NAc.

Moreover, the available results suggest that contingent and non-contingent cocaine procedures produce opposite effects on GABAergic transmission in the NAc. Using a non-contingent repeated procedure, Kalivas et al. demonstrate that the basal (ambient) levels of GABA in the NAc are increased after 3-week withdrawal from cocaine administration, which may result from the potential hyperactivity of fast-spiking interneurons (FSIs) (Xi et al., 2003). Our recent results indicate that the membrane excitability of CB1-expressing FSIs within the NAcSh is increased after withdrawal from cocaine exposure, which may lead to increased release of GABA (Winters et al., 2012). On the other hand, a decreased level of GABA in the NAc is observed after extinction from cocaine self-administration (Miguéns et al., 2011).

Interestingly, our present results (Figures 12 & 14) also indicate the opposite alterations in GABAergic transmission to the NAc following contingent versus non-contingent exposure to cocaine. A challenge i.p. injection of cocaine after 21 days withdrawal shifts the chloride reversal potential to the depolarized direction, whereas the contingent re-exposure to cocaine after 21 days withdrawal altered the chloride reversal potential to the hyperpolarized direction. Thus, GABAergic synaptic transmission in the NAc might be one of the neural substrates that differentiate the cellular and behavioral effects of contingent and non-contingent cocaine procedures.

Future direction

Research for investigating the role of long-range VTA-to-NAc GABAergic projection and its interaction to the DA projection has just started recently. Our results show the important interaction between VTA-to-NAc DAergic and GABAergic projections (Chapter 3) and the dynamic alterations in the GABAergic transmission during cocaine withdrawal (Chapter 2, 3 & 4). These results indicate the time-dependent involvement of GABAergic transmission in the addiction-related behaviors. However, our understanding about drug-induced synaptic adaptation is still far from a “masterpiece” and further investigation is definitely necessary.

- *Heterogeneous cell types*

As briefly described, NAc MSNs are highly heterogeneous and can be divided into different subpopulations. For example, based on receptor/transmitter subtypes, the NAc neurons can be divided into three subpopulations, one co-expressing DA D1 receptors and substance P, one co-expressing DA D2 receptors and enkephalin, and one expressing both D1

and D2 receptors (Gong et al., 2003; Le Moine & Bloch, 1995; K.-W. Lee et al., 2006). Based on electrophysiological properties, NAc neurons can be categorized as neurons with or without gap junctions with their neighboring NAc neurons (O'Donnell & Grace, 1993). Thus, a key future direction would be to determine whether these identified cocaine-induced adaptations are cross-board effects or only occur in one subtype of neurons.

- *Long-term inhibitory plasticity*

The finely tuned balance between the excitatory and inhibitory synaptic inputs is important for the neuronal stability. We hypothesize that disruption of this balance is one way through which cocaine reshapes the basal functional state of NAc MSNs. As many studies have suggested, the long-term plasticity at excitatory synapses in NAc after cocaine experience is impaired (Fourgeaud et al., 2004; C.-C. Huang, Liang, Lee, & Hsu, 2014; Martin, Chen, Hopf, Bowers, & Bonci, 2006). Further investigation at inhibitory synapses in the NAc, in addition to the DA-triggered iLTD within the VTA-to-NAcCo projection, will be necessary because excitatory and inhibitory synaptic plasticity can exist concurrently and the heterosynaptic nature of induction of inhibitory synaptic plasticity may play an important role to regulate the balance between excitatory and inhibitory synaptic transmission (Castillo et al., 2011).

- *Impact of membrane excitability*

In addition to focusing on the synaptic inputs, another important factor that determines the functional output of NAc MSNs is the membrane excitability, which also warrants further investigation. Our previous computational analysis predicts dynamical alterations of functional output of NAc MSNs after cocaine experience (Mu et al., 2010). However, the computational

prediction is not consistent with the actual input-output efficacy (Fig. 16). Because of limited availability of data on specific withdrawal points, Mu's study could only use SK channel-related data (Ishikawa et al., 2009) for the membrane excitability, although other ionic conductances (such as sodium, potassium, and calcium currents) also play important roles in the membrane excitability. It has been reported that the number of these ionic conductance seems to be involved in cocaine-induced membrane adaptation (Hu, Basu, & White, 2004; Kourrich et al., 2013; Zhang, Cooper, & White, 2002; Zhang, Hu, & White, 1998), and further investigation in those ionic conductances, together with other cocaine-induced adaptations and hSMP, will help understand the drug-induced motivational alterations.

- *GABAergic projection from the VTA to the NAc*

The discrepancy found in the VTA-to-NAc GABAergic projection (M. T. Brown et al., 2012; van Zessen et al., 2012) requires further investigation. Our electrophysiological recordings are performed in the caudal part of the NAcCo. Although we did not examine CINs, we recorded reliable response in MSNs in this specific NAc region. Accumulating evidence shows that the different population of VTA DAergic neurons project to different NAc sublocations (Hnasko, Hjelmstad, Fields, & Edwards, 2012; Lammel et al., 2008; Lammel, Ion, Roeper, & Malenka, 2011; Walsh & Han, 2014). Also, some studies show that the inhibition of different parts of the NAc leads to opposite appetitive behavioral outcomes (Volman et al., 2013). These results indicate the strong heterogeneity within the NAc. Further investigation is needed to determine whether this kind of heterogeneity applies to the long-range GABAergic neurons.

Concluding Remarks

Mainly focusing on GABAergic transmission in the NAc, my thesis work provides some new insights to the field. Our results show a novel form of iLTD within the parallel projections of DAergic and GABAergic terminals from the VTA to the NAcCo. We also demonstrate the dynamic regulation of GABAergic transmission after cocaine experience. These adaptive changes in inhibitory transmission, together with alterations in excitatory transmission and membrane excitability, may contribute to the pathophysiological behaviors observed in cocaine-exposed individuals. Once the overall effects on the output of NAc MSNs by cocaine is revealed, the identified components, including GABAergic transmission, may lead to the design of treatments for drug seeking and drug taking.

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