COCAINE SELF-ADMINISTRATION:

ADAPTATIONS TO THE GLUTAMATERGIC SYSTEM AND

CONSEQUENCES FOR OFFSPRING EMOTIONAL CONTROL

Samantha Louise White

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Supervisor of Dissertation

Signed: _____

R. Christopher Pierce, Ph.D., Associate Professor of Neuroscience in Psychiatry

Graduate Group Chairperson

Signed: _____

Joshua I. Gold, Ph.D., Associate Professor of Neuroscience

Dissertation Committee

Kelly L. Jordan-Sciutto, Ph.D., Chair and Associate Professor of Pathology (Committee Chair)

Julie A. Blendy, Ph.D., Professor of Pharmacology

Robert G. Kalb, M.D., Professor of Neurology

Jose Moron-Concepcion, Ph.D., Assistant Professor of Anesthesiology (External Member)

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ABSTRACT

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ADAPTATIONS TO THE GLUTAMATERGIC SYSTEM AND

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Samantha Louise White

R. Christopher Pierce, Ph.D.

Cocaine abuse and relapse remain a major public health concern in the United States and worldwide for which there is currently no approved pharmacotherapeutic intervention. Rodent cocaine self-administration, extinction, and priming-induced reinstatement can be used to model human cocaine seeking. A growing body of evidence indicates that the transport and stabilization of calcium-permeable (CP) AMPA glutamate receptors to synapses in the accumbens, a process involving CaMKII, is associated with the reinstatement of cocaine seeking. Additional evidence indicates that the dorsal striatum contributes to aspects of cocaine addiction. Moreover, relapse to cocaine abuse has been connected to elevated levels of anxiety during withdrawal and anxiolytic agents decrease the latency for animals to self-administer cocaine. A growing body of evidence indicates that environmental information can be inherited. We have previously described a cocaine-resistance phenotype in the offspring of animals that have self-administered cocaine. The enhancement of cocaine's anxiogenic effects may contribute to reduced cocaine self-administration among male cocaine-sired rats. Here, a variety of behavioral, cellular, molecular, and electrophysiological techniques are used to examine how cocaine experience directly affects the glutamatergic system in the

dorsal striatum and accumbens, as well as its indirect consequences for drug-naïve offspring. Acute exposure to cocaine in drug naïve rats increased CaMKII-mediated phosphorylation of GluA1-containing AMPA receptors in the DL striatum, an effect that was not observed during cocaine priming-induced reinstatement of drug seeking. The increased phosphorylation of CaMKII and GluA1 following acute cocaine may be a compensatory mechanism in the DL striatum. Accumbens shell CP-AMPAR receptor transmission, mediated through interactions of GluA1-containing AMPARs with accessory protein SAP97, is necessary for cocaine reinstatement. Consideration of GluA1 subunit accessory proteins as potential novel targets for pharmacotherapeutic interventions in cocaine craving and addiction is warranted. Male offspring of cocaine-experienced sires exhibit baseline anxiety-like behaviors that are unaltered by subsequent cocaine exposure and dysregulation of hippocampal cellular and molecular correlates of anxiety. This identifies impairments of male offspring emotional control due to sire cocaine exposure independent of the cocaine-resistance phenotype. Collectively, these findings advance our knowledge of the direct and intergeneration effects of cocaine experience on the brain and behaviors.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 3

Chapter 4

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

PREFACE

COCAINE ADDICTION: A PUBLIC HEALTH CONCERN

Samantha L. White

Substance abuse is defined as a maladaptive, recurrent pattern of substance use that significantly impairs an individual's daily functioning and/or interpersonal relationships. The point when an individual fails to successfully stop drug use, displays symptoms of drug tolerance (requiring progressively higher doses to achieve the desired effect), or withdrawal (adverse psychological and/or physiological symptoms due to abstinence from the drug), is indicative of dependence or addiction (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). There are three categories of abused substances: psychostimulants, depressants, and hallucinogens, which have a variety of rewarding effects when used that contribute to their addiction liability. These effects promote continued abuse despite negative consequences to health, career, and interpersonal relationships, influencing the lives of more than just the addicted individual (4th ed., text rev.; *DSM–IV–TR*; American Psychiatric Association, 2000). This dissertation will focus on the neurobiological, behavioral, and inherited effects of one psychostimulant in particular: cocaine.

The United States spends approximately 181 million dollars annually to combat the use of illicit drugs, including cocaine (Karch, 1999; SAMHSA, 2012). In fact, the United States is the current leader in global demand for cocaine. Cocaine use is

involved in almost 500,000 emergency room visits annually, and over 600,000 people begin using cocaine each year (NIDA, 2009; SAMHSA, 2012; NADCP, 2013). Cocaine abuse is positively correlated with the incidence of fatal road accidents, the spread of HIV/AIDS and hepatitis, and developmental delays, among myriad other negative effects (NIDA, 2010; Elvik, 2012). Unfortunately, cocaine abuse is also associated with a high rate of relapse to drug taking following periods of abstinence, making cocaine addiction a continuing public health concern that impacts our country's communities, the government, and medical system (O'Brien, 1997). Although efforts have been made by society, the government, and the medical field to cope with cocaine addiction and relapse, there remains no permanent behavioral or pharmacotherapeutic treatment for this disorder.

Society

In the relatively recent past, drug addiction, once considered a moral failing by many in our society, has been redefined as a medical condition stemming from long-lasting changes to brain structure and chemistry (Sowers, 1998; Martinez et al., 2007). Given that many recognize that an addict's first experience with a drug such as cocaine is typically voluntary, research showing lasting alterations in brain function has encouraged, at best, a reluctant public acceptance of cocaine dependence as a biological deficit requiring medical intervention (Childress et al., 1999; Hyman et al., 2006; Volkow et al., 2009). In fact, self-reported addicts still describe difficulty stopping use and/or increased incidence of relapse due to concerns that withdrawal symptoms will negatively impact job opportunities and increase the risk for stigmatization by their community (Mooney et al., 2012). Because relapse rates inversely correlate with one's level of education and employment, understanding and support by the general public is

paramount to the success of those battling addiction in a period rife with school closings and spiking unemployment rates (SAMHSA, 2012; Statistics, 2013). Given that continued drug abuse and relapse increase the socioeconomic burden for non-drug users, and current efforts to treat addiction are only impeded by prejudiced views of addiction, it is in the best interest of the public to support policies that advance research on treatment of addiction as a medical condition (Dackis et al., 2005; Dackis and O'Brien, 2005).

Government

Government policies, across executive, legislative, and judicial levels, have recently started to address drug addiction in a manner that educates the public and helps advance treatment research. In 2008, President Obama created the Office of National Drug Control Policy in an effort to counter the failed “war on drugs” crusade of previous administrations; this act established dedicated funds for substance abuse treatment programs through 2015 (ONDCP, 2013). The National Drug Control Strategy created by this office relies upon scientific research indicating a biological basis for addiction to help form public health policy that has successfully reduced drug use and its consequences. Countless movements, ranging from novel treatment programs to budgeting for addiction rehabilitation and health care, to improvements for prisoners with a history of substance abuse, have come before state legislatures in the past decade (NCSL, 2013). One benefit of adjustments at the executive and legislative levels can be quantified in the judicial system, where for every dollar spent on substance abuse treatment, seven dollars are saved in criminal justice system costs (SAMHSA, 2012). Additionally, reform of the judicial branch created drug courts: special dockets designed specifically for non-violent offenders with a history of substance abuse that allow for continued, court-

managed participation in treatment programs to help reduce the rate of relapse associated with jail-time and recent parole (www.nadcp.org; SAMHSA 2010). These actions suggest a growing government awareness and acceptance of drug addiction as a disease, which should both decrease lingering stigma in the minds of the general public and ensure that the medical system and researchers obtain the support and funding necessary to further treatment options.

Science and Medicine

Though the scientific and medical communities lead the efforts to define and treat substance abuse as a disease, there remain a multitude of roadblocks in translating findings to practice. Despite the inclusion of substance abuse rehabilitation as an essential component of health care in the 2010 Affordable Care Act, most addicted individuals report paying out-of-pocket for treatment programs and many health insurance companies still do not offer coverage for cocaine addiction (Mooney et al., 2012; SAMHSA, 2012; ONDCP, 2013). Unlike nicotine, alcohol, or heroin dependence, there are no FDA-approved pharmacotherapeutic interventions for cocaine addiction, leaving time-consuming behavioral therapies like cognitive behavioral therapy as the only alternatives (Penberthy et al., 2010). One attractive proposal that has reached stage II clinical trials is the TA-CD cocaine vaccine, which promotes the development of antibodies that bind molecules of cocaine before they can bind to receptors in the body, blunting the rewarding effects of the drug (Haney et al., 2010). Unfortunately, this prospective solution is not without flaws. Medically, it takes weeks of shots for cocaine-specific antibodies to build up to therapeutic levels, booster injections are required to maintain effectiveness past 2 months, and individuals risk serious injury or death if they attempt to overwhelm the antibody response by taking high quantities of cocaine (Haney

et al., 2010; Young et al., 2012). Ethical issues arise when contemplating who should receive the vaccine, production costs versus ability to afford the injections, prophylactic use in at-risk groups, and the potential stigma associated with use of the vaccine (Hall and Gartner, 2011; Young et al., 2012). In spite of all that scientific research has done to amend policies on drug addiction and to sway public opinion, more work is needed to ensure appropriate consideration of this disease in the health care system as well as establishing safe, effective interventions that stop the cycle of abuse and relapse.

Despite the changing perception and management of substance abuse disorders by society and the government and evidence that investments in treatment programs and biomedical research save money long-term, more must be done to address this public health issue. Over 19 million people still need specialized treatment for addiction and increased globalization of cocaine trafficking suggests that use will continue regardless of containment policies (Costa Storti and De Grauwe, 2009; Mooney et al., 2012). Much of the scientific evidence that has provided insight into the nature of addiction and has prompted a shift in public opinion of this disease has stemmed from preclinical research (Dackis and O'Brien, 2005). The preclinical experiments outlined in this dissertation aim to better understand the neurological mechanisms underlying, and consequences of, cocaine addiction and relapse with the hope of identifying a novel target for future pharmacotherapeutic intervention.

CHAPTER 1

GENERAL INTRODUCTION

Samantha L. White

Animal Models of Addiction: Cocaine Self Administration and Reinstatement

To delve into the underlying mechanisms of human cocaine addiction and relapse, scientists have relied upon well-established preclinical rodent and non-human primate animal models. In rats, cocaine self-administration, extinction, and reinstatement of cocaine-seeking behavior represent the cycle of addiction, recovery, and relapse seen in humans (de Wit and Stewart, 1981; Shaham et al., 2003). The drug-free extinction period loosely models the times when human addicts are enrolled in rehabilitation centers or are otherwise no longer exposed to cocaine, as true cocaine extinction learning does not typically occur in humans (Epstein et al., 2006). As with human relapse, three factors precipitate the reinstatement of drug seeking in rodents: stress exposure (a mild foot shock or forced-swim test), environmental stimuli associated with the drug (often a light and/or tone paired with drug infusions), or re-exposure to the drug itself (non-contingent, experimenter delivered, intra-peritoneal ["i.p."] injections) (Schmidt and Pierce, 2010).

In the rat model, rodents are implanted with jugular catheters and are trained to self-administer cocaine intravenously (i.v.) in an operant chamber (Spealman and Goldberg, 1978). Typically, this requires the rodents to learn to press one lever, the "active" lever, in an established schedule in order to receive the drug. The animals must also learn that pressing a second, "inactive" lever, has no consequence on drug delivery. The schedule of reinforcement can range from a fixed ratio (FR), where the amount of work required to obtain a single infusion of the drug remains constant (e.g. one lever press results in one infusion, 5 lever presses results in one infusion), to a progressive ratio (PR), where animals must respond with an increasing frequency in order to receive the same amount of drug (Spealman and Goldberg, 1978). The extent of self-

administration also varies in both the amount of cocaine available within a given session as well as the total number of self-administration sessions. Short-access paradigms generally use sessions 2 hours or less in length, while long-access or "binge" paradigms employ sessions that are 4-6 hours long (Pierce and Wolf, 2013; Purgianto et al., 2013). Regardless, following self-administration training, animals either remain in their home cage for some period of time, or they participate in extinction sessions where lever pressing no longer results in drug administration. During extinction training, rodents will initially seek cocaine by accessing the active lever at a very high rate before gradually tapering off the behavior upon learning the drug is no longer available. This excessive drug-seeking behavior when drug is initially absent is known as an "extinction burst," and drug-seeking behavior is considered extinguished once responding is significantly reduced (for example, by 15%) from that on the last day of self-administration. Re-exposure to the drug, also known as cocaine priming-induced reinstatement, typically produces the most robust, lasting drug seeking response in rodents (Shalev et al., 2002). While a range of doses can be used during the reinstatement of cocaine seeking, the minimum dose to reliably elicit drug-seeking behavior is 10 mg/kg (i.p.) (Anderson et al., 2003).

Cocaine and Neurotransmitter Systems: Dopamine and Glutamate

Dopaminergic System

Cocaine prevents the reuptake of all three monoamines (dopamine, serotonin, and norepinephrine) in the brain, resulting in disproportionately increased extracellular concentrations of these neurotransmitters (NIDA, 2011). The reinforcing properties of cocaine are primarily reinforced by dopaminergic transmission (Pierce and Kumaresan, 2006). Increased extracellular dopamine is associated with the reinforcing effects of

cocaine and dopamine reuptake inhibitors are sufficient to induce the reinstatement of cocaine seeking without re-exposure to the drug itself (Schmidt et al., 2005; Pierce and Vanderschuren, 2010). Dopamine receptors (of which there are 5 subtypes) are G-protein coupled receptors that are categorized into two groups, which can have opposing consequences on intracellular signaling following activation. D1-like receptors (D1R) couple to a $G\alpha_s$ or $G\alpha_{olf}$ protein to increase cAMP production in the cell, while D2-like receptors (D2R) couple to a $G\alpha_i$ or $G\alpha_o$ protein to decrease cAMP production (Neve et al., 2004). That said, neuronal responses due to the activation of D1-like or D2-like receptors vary widely, suggesting a modulatory role for dopamine in the brain.

In the ventral striatum, particularly in the nucleus accumbens (NAc), phasic dopamine receptor activity in medium spiny neurons (MSNs) occurs during the acquisition of cocaine self-administration (Willuhn et al., 2012). Further, cocaine exposure causes the potentiation of NAc D1R MSNs in parallel with locomotor sensitization, and recent work has shown that activation or inhibition of D2Rs in the accumbens suppresses cocaine self-administration or increases the motivation to acquire drug, respectively (Pascoli et al., 2011; Bock et al., 2013). Increased dopamine transmission through dopamine receptors has been connected to the priming-induced reinstatement of cocaine seeking (Schmidt et al., 2005; Shaham et al., 2003). Furthermore, D1- and D2-like agonist compounds promote reinstatement in rodents and non-human primates (Khroyan et al., 2003; Bachtell et al., 2005; Schmidt and Pierce, 2006). Injection of D1R or D2R antagonists into the shell subregion of the accumbens attenuates cocaine self-administration without altering food-seeking behavior, suggesting a role for both receptors in the reinforcing efficacy of cocaine (Bari and Pierce, 2005). However, the same antagonists injected into the core subregion

attenuate both the reinstatement of cocaine- and food-seeking behavior, indicating a global influence on reward in this nearby area.

Glutamatergic System

Though the excitatory neurotransmitter glutamate is not directly impacted by the presence of cocaine, repeated cocaine exposure changes the glutamatergic tone of the mesocorticolimbic reward system. Following cocaine exposure, there is increased extracellular dopamine in the medial prefrontal cortex (mPFC), which in turn activates the glutamatergic pathway from the mPFC to the accumbens subregions, leading to altered accumbal glutamate following repeated cocaine exposure and during reinstatement (Pierce et al., 1996; Park et al., 2002; McFarland et al., 2003). In fact, cocaine priming-induced reinstatement selectively increases NAc extracellular glutamate, and transient inactivation of mPFC efferents using gamma-aminobutyric acid (GABA) agonists prevents both the reinstatement of cocaine seeking and the rise in glutamate in the NAc (McFarland et al., 2003).

When released, glutamate, a major excitatory neurotransmitter, binds both ionotropic receptors, such as α-Amino-3-hydroxy-5-Methylisoxazole-4-Propionic Acid (AMPA) receptors or *N*-methyl-D-aspartate (NMDA) receptors, and metabotropic glutamate receptors (mGluRs) (Pierce and Schmidt, 2010). While the consequences of NMDAR activation during cocaine self-administration and cocaine priming-induced reinstatement are complex, there is a much clearer role for glutamatergic transmission via AMPARs (Pierce and Schmidt, 2010; Pierce and Wolf, 2013). The activation of D1Rs in the accumbens shell initiates a signaling cascade that stimulates L-type calcium channels and produces serial phosphorylation of calcium calmodulin-dependent protein kinase kinase α (CaMKII) and the phosphorylation and trafficking of GluA1 subunit of

AMPA glutamate receptors to the shell plasma membrane (See Figure 1.1) (Anderson et al., 2008). CaMKII can maintain an activated state via autophosphorylation at threonine 286, allowing continued phosphorylation of AMPAR subunit GluA1 at serine 831 even after D1R signaling has ceased (Miller et al., 1988; Lisman and Zhabotinsky, 2001). Disrupting the function, activation, or trafficking of any of these proteins – L-type calcium channels, CaMKII, or GluA1 in the shell – attenuates cocaine priming-induced reinstatement, which is presumably a result of increased extracellular glutamate acting on AMPARs (Anderson et al., 2008).

Brain Regions Involved during the Reinstatement of Cocaine Seeking

Cocaine exerts its actions within multiple brain regions that comprise the mesocorticolimbic reward system (Ritz et al., 1990; Schmidt and Pierce, 2010). This circuitry encompasses dopaminergic neurons in the ventral tegmental area (VTA), limbic structures like the NAc, and cortical areas including the prefrontal cortex (PFC) (Feltenstein and See, 2008). Typically, these regions evolved to identify natural rewards like food or sex, but cocaine disproportionately enhances activation of the system through changes to neuronal firing patterns, cellular and molecular adaptations, altered biochemistry, or some combination of all three (Olsen, 2011; Pierce and Wolf, 2013).

Ventral Tegmental Area

The ventral tegmental area (VTA) is located in the midbrain and contains one of the largest populations of dopamine-producing neurons in the brain. These neurons send projections to other areas in the mesocorticolimbic reward pathway including the NAc and PFC, and connected structures like the amygdala and hippocampus (Figure 1.2) (Mameli and Luscher, 2011). Interestingly, the accumbens and the PFC send inhibitory and excitatory projections to the VTA, respectively, creating a feedback loop to modulate

reward learning (Sesack and Grace, 2010). Approximately 60% of VTA neurons are dopaminergic and contain excitatory glutamate receptors that increase neuronal excitability and neurotransmitter release upon activation (Bayer and Glimcher, 2005). The firing pattern of these dopamine neurons switches from tonic to phasic firing upon presentation of appetitive stimuli or, if the stimuli have been paired with a visual or auditory cue, upon presentation of the cue, providing a neuronal correlate of reward prediction (Schultz et al., 1997). Approximately 35% of all neurons in the VTA GABA-containing inhibitory interneurons, the activation of which prevents the release of dopamine (Nair-Roberts et al., 2008). As a result of cross-talk between these neuronal populations and outside inputs, dopamine neurons in the VTA undergo synaptic plasticity upon exposure to drugs of abuse (Sesack and Grace, 2010; Mameli and Luscher, 2011). Acute exposure to cocaine is sufficient to promote an increase in calcium-permeable AMPA receptors in VTA neurons that results in transient long-term potentiation (LTP), a form of synaptic plasticity often associated with learning and memory (Bellone and Luscher, 2006). In the case of cocaine self-administration, LTP is measured in VTA dopaminergic neurons for months following the last infusion of cocaine, which may represent a cellular correlate underlying addictive behavior (Chen et al., 2008a).

Prefrontal Cortex

The prefrontal cortex, and the mPFC in particular, receives dopaminergic projections from the VTA and sends excitatory glutamatergic projections back to the VTA as well as to other regions of the reward pathway like the NAc (Figure 1.3) (Sun and Rebec, 2005). In fact, the connections between the mPFC and the accumbens are required during cocaine priming-induced reinstatement (Del Arco and Mora, 2008; Vassoler et al., 2008).

Administration of a dopamine receptor agonist directly into the PFC can elicit the reinstatement of drug-seeking behavior even when the VTA is inactivated (McFarland and Kalivas, 2001). Tracing studies have identified that the projections between the mPFC and the NAc can be separated along the mPFC dorsal (d) to ventral (v) axis, with successful attenuation of cocaine priming-induced reinstatement via disruptions to dopamine signaling in the dmPFC (McFarland and Kalivas, 2001; Porrino et al., 2004; Schmidt et al., 2005). Further, the interaction of these brain regions provides a key connection between the directly influenced dopamine system and the indirect modulation of the glutamatergic system. Activation of dopamine receptors in the PFC via dopamine efferents from the VTA increases the surface expression of cortical GluA1 AMPA receptor subunits, facilitating excitation and LTP in the PFC (Sun et al., 2005). The probability of glutamate release from the PFC onto accumbens MSNs increases with both non-contingent and, to a greater degree, contingent cocaine exposure (Suska et al., 2013). Transient, pharmacological inactivation of the dmPFC attenuates priming-induced reinstatement of cocaine seeking as well as the associated rise in extracellular glutamate in the accumbens core (McFarland et al., 2003). Together, these data represent a mechanism through which cocaine exposure promotes synaptic plasticity in the PFC to modulate the mesocorticolimbic reward system during drug use.

Nucleus Accumbens

Located in the ventral striatum, the nucleus accumbens processes drug and natural rewards and can be separated into two subregions, the core and the shell (Schmidt and Pierce, 2010). The neuronal population is relatively homogenous across both subregions, comprised mainly of GABAergic medium spiny neurons (MSNs) (Tepper et al., 2007). These MSNs contain dopamine receptor (R) subtypes (D1-like and D2-like)

as well as glutamate receptors (including AMPARs) that activate in response to dopaminergic and glutamatergic projections from the VTA and PFC, respectively (Figures 1.1 and 1.3) (Sesack and Grace, 2010). Together, these subregions comprise the primary input nuclei of the limbic system and the basal ganglia (Heimer et al., 1991). The core receives projections from the dmPFC, while the shell receives projections from the vmPFC (Figure 1.3) (Schmidt et al., 2005). The core is associated with the basal ganglia, sending inhibitory projections via the ventral pallidum and substantia nigra to motor areas of the cortex, and receives dopaminergic efferents from the VTA after processing occurs in the shell (Day and Carelli, 2007; Pierce and Vanderschuren, 2010). On the other hand, the accumbens shell is associated with the limbic system, is first to receive direct dopaminergic input from the VTA, and projects back to the VTA in addition to other subcortical limbic structures (See Figure 1.4) (Haber et al., 2000; Day and Carelli, 2007).

While both regions are important during cocaine self-administration and the reinstatement of drug-seeking behavior, their roles are distinct. Thus, the core is involved in the acquisition of drug seeking and cue-induced cocaine seeking, as well as broader, non-drug operant learning, while the shell is the primary mediator for the reinforcing effects of cocaine (Ito et al., 2004; Bari and Pierce, 2005; Di Ciano et al., 2008). Transient inactivation of neurons in either the core or shell subregion of the accumbens significantly attenuates cocaine priming-induced reinstatement, while intra-accumbal microinjection of AMPA alone reinstates cocaine-seeking behavior (Ping et al., 2008; Vassoler et al., 2008). Further, injection of D1R or D2R agonists into the accumbens elicits the reinstatement of drug seeking, and D1R activation is associated with increased AMPAR expression during cocaine-priming induced reinstatement (Bachtell et al., 2005; Schmidt et al., 2006; Anderson et al., 2008). Interestingly,

increased surface expression of calcium permeable AMPARs occurs after extended abstinence as well as during cocaine priming-induced reinstatement (Pierce and Wolf, 2013).

Dorsal Striatum

While the accumbens in the ventral striatum has clear connections to the rewarding efficacy of cocaine, the dorsal lateral (DL) striatum has been implicated in the development of habitual drug seeking (Pierce and Vanderschuren, 2010). Habit-based responding is independent of the value of the reinforcer and relies on a stimulus-response association (Dickinson et al., 2002). Conversely, goal-directed responding encompasses behaviors or actions that are mediated by the value of a given reinforcer (Miles et al., 2003). The ventral striatum is more consistently associated with goal-directed behaviors, and the dorsal striatum is more involved in habitual responding. With limited cocaine self-administration training, negative reinforcers (like foot shock) suppress drug-seeking behavior, and blocking dopaminergic transmission in the NAc, but not DL striatum, decreases the rewarding efficacy of cocaine (Vanderschuren and Everitt, 2004; Bari and Pierce, 2005; Murray et al., 2012). Interestingly, inactivation of the dorsomedial (DM) striatum also prevents the acquisition of operant behaviors, and dopamine transmission is required for goal-directed cocaine seeking (Yin et al., 2005; Murray et al., 2012). Following prolonged periods of cocaine self-administration experience or cue-controlled self-administration, negative reinforcers no longer influence drug seeking, and intra-DL striatum dopamine or AMPAR antagonists attenuate responding (Vanderschuren and Everitt, 2004; Vanderschuren et al., 2005).

A study in primates used antero- and retrograde tracers to establish an ascending spiral of connectivity from dopamine neurons in the midbrain across the

nucleus accumbens, dorsomedial (DM) striatum, and dorsolateral (DL) striatum (Figure 1.4) (Haber et al., 2000). Similar to the accumbens, dorsal striatal brain regions also contain GABAergic MSNs that are phasically active, firing in anticipation of movements, particularly those associated with reward (Nicola, 2007). There is increased modulation of DM and DL MSN firing, as well as increased MSN excitation and synaptic strength, during early and late stages of operant training, respectively (Yin et al., 2009). During the acquisition of cocaine-seeking behavior, dopaminergic processing first occurs in the ventral striatum, sending both feed-forward GABAergic inhibitory projections to the DM and DL striatum and feedback to the midbrain dopamine neurons (Porrino et al., 2004; Pierce and Vanderschuren, 2010). As rats self-administer cocaine, phasic dopamine release increases in the DL striatum over time, but only after initial dopaminergic activation in the ventral striatum (Willuhn et al., 2012). Activation of DL striatum MSNs is necessary for the resistance to reinforcer devaluation seen in habit-based operant behaviors (Everitt et al., 2008). Unlike during the acquisition of drug-seeking behavior, as cocaine seeking/taking becomes compulsive, extracellular glutamate increases in the DL striatum and infusion of a dopamine receptor antagonist will attenuate responding (Veeneman et al., 2012).

Interestingly, a small but growing body of literature also identifies involvement of the DL striatum prior to the point at which drug-seeking behavior is considered habitual. Reversibly inactivating this region during the acquisition and maintenance of cocaine seeking, periods when the reinforcing value of the drug mediates behavior, attenuates drug seeking (Pacchioni et al., 2011; Veeneman et al., 2012). In the DL striatum, limited, experimenter-delivered cocaine increases glutamatergic transmission, and inactivation of the DL striatum attenuates cocaine-priming and cue-induced reinstatement of drug seeking (McKee and Meshul, 2005; Gabriele and See, 2011). To

extend this work, chapter two of this dissertation investigates the biochemical changes that occur in the DL striatum during initial cocaine exposure, short-access self-administration, and priming-induced reinstatement of cocaine seeking.

AMPA Glutamate Receptors and Cocaine

The excitatory, ionotropic AMPA glutamate receptors contain four subunits, GluA1-GluA4, that form a pore to allow the passage of sodium, potassium, and occasionally calcium ions after glutamate binding (Bear, 2007). GluA2-containing AMPA receptors are typically calcium impermeable due to GluA2 Q/R editing of the pore-containing region of the subunit (Seeburg, 1996). Consequently, GluA2-lacking AMPA receptors allow the influx of calcium upon glutamate binding, leading to an enhanced excitatory post-synaptic response (Jonas and Burnashev, 1995). Inward rectification, a reduction in current as membrane voltage increases, provides electrophysiological evidence of increased calcium-permeable GluA2-lacking AMPARs. Approximately 10% of accumbal AMPA receptors are calcium-permeable (CP) in drug-naïve rodents, with the vast majority being comprised of GluA1/GluA2 subunits (Wolf and Tseng, 2012). However, chronic cocaine exposure followed by 1 month of withdrawal increases accumbal GluA1 subunit surface expression and produces a 30% increase in rectification index, suggesting that these subunits form CP-AMPARs (Conrad et al., 2008). Confirming that withdrawal after chronic cocaine use increases the levels of CP-AMPARs in the accumbens, Conrad (2008) illustrates that AMPAR-mediated evoked excitatory post-synaptic potentials (eEPSCs) of accumbal MSNs are reduced by the CP-AMPAR antagonist 1-naphthyl acetyl spermine (Naspm).

Cocaine priming-induced reinstatement is associated with increased GluA1 subunit phosphorylation, trafficking, and insertion of GluA1 AMPA receptors into the

accumbens shell cell surface (Anderson et al., 2008). Phosphorylation of GluA1 subunits occurs at multiple residues during cocaine exposure, but phosphorylation by CaMKII at serine 831 increases the single-channel conductance and efficiency of AMPA receptor conformational change upon glutamate binding, particularly in the presence of transmembrane AMPA receptor accessory proteins (TARPs) (Kristensen et al., 2011; Lisman et al., 2012). It is important to note, however, that CaMKII, phosphorylation of GluA1-ser831, and increased GluA1 surface expression during priming-induced reinstatement are transient relative to that seen after extended withdrawal from long-access self-administration (Pierce and Wolf, 2013). Regardless, none of these receptor changes occur following experimenter-delivered, non-contingent drug injections – self-administration experience, and presumably the maladaptive learning inherent to developing cocaine addiction, is necessary (McCutcheon et al., 2011).

As illustrated in Figure 1.1, stimulation of mGluRs and D1Rs following cocaine exposure activates protein kinases C and A, respectively, in addition to CaMKII (Wolf et al., 2003; Chen et al., 2007; Schmidt and Pierce, 2010). In turn, protein kinase C (PKC), activated in the intracellular signaling cascade of group 1 mGluRs, also phosphorylates GluA1 at serine 831, as well as serines 816 and 818, which enhances GluA1 binding to scaffolding protein 4.1N (Lin et al., 2009; Olive, 2009). Simultaneously with GluA1 phosphorylation during cocaine priming-induced reinstatement, PKC phosphorylates GluA2 subunits at serine 880, altering their interactions with GluA2-specific scaffolding proteins to promote internalization of these subunits and potentially contributing to the transient shift towards more CP-AMPARs (Famous et al., 2008; Pierce and Wolf, 2013). Protein kinase A, PKA, phosphorylates GluA1 at serine 845, leading to insertion of the receptor subunit and increased surface expression in conjunction with serine 831 phosphorylation (Sun et al., 2008). However, increased serine 845 phosphorylation has

only been measured following withdrawal from long-access self-administration, and not during cocaine priming-induced reinstatement (Anderson et al., 2008; Ferrario et al., 2011b). Together, these findings suggest that cocaine-mediated kinase phosphorylation of AMPAR subunits, particularly GluA1, underlies changes in receptor composition at the plasma membrane surface.

GluA1-containing AMPAR Accessory Proteins: 4.1N and SAP97

Cocaine-mediated changes in surface receptor expression would not be possible without accessory proteins to aid in the trafficking and stabilization of GluA1 subunits (Anggono and Huganir, 2012; Lisman et al., 2012). Changes in the trafficking and anchoring of GluA1-containing AMPARs to the cell surface mediate the increased glutamatergic plasticity in accumbens MSNs following cocaine self-administration and during cocaine priming-induced reinstatement. On its own, GluA1 is unable to associate with a primary scaffolding protein at the post-synaptic density (PSD), PSD-95, requiring various other accessory proteins to secure GluA1-containing AMPA receptors at and around the synapse (Anggono and Huganir, 2012). While CaMKII directly phosphorylates GluA1 at serine 831 as part of this process, it also regulates AMPAR trafficking indirectly by phosphorylating proteins like synapse-associated protein 97 (SAP97) that help to anchor GluA1 at the PSD (Nikandrova et al., 2010; Opazo et al., 2010). In addition to PSD95, GluA1 subunits are stabilized in synapses against the actin-spectrin cytoskeleton by another accessory protein, 4.1N (See Figure 1.1) (Shen et al., 2000).

SAP97

Despite the fact that GluA1 does not directly bind PSD-95, the C-terminal domain of GluA1 contains a PDZ (PSD95-Discs large-ZO-1) binding motif that associates with the second PDZ domain of SAP97 (Cai et al., 2002). Like GluA1, SAP97 complexes with

CaMKII and is phosphorylated at serines 39 and 232, and over-expression of this protein can rescue GluA1 subunit trafficking in a double knockout of PSD95/93, offering an alternative mode of stabilization at the plasma membrane (Nikandrova et al., 2010). Deletion of the last 4 amino acids of GluA1 prevents interaction with SAP97 and reduces GluA1 surface expression, and a point mutation in GluA1 changing threonine 887 to an alanine also blocks SAP97 binding and slows GluA1 subunit insertion at the cell surface (Passafaro et al., 2001). Over-expression of SAP97 in cultured neurons increases the level of synaptic AMPARs, as well as their associated miniature evoked post-synaptic currents, an effect that is dependent on the ability of SAP97 to bind 4.1N (Rumbaugh et al., 2003). That said, there is also evidence that while SAP97 is involved in GluA1 trafficking and retention at the membrane, it is not required (Howard et al., 2010). Over-expression of SAP97 in HEK293 cells that also express GluA1 has no effect on subunit movement (Cai et al., 2002). Further, while SAP97 over-expression enhances the formation of dendritic spines and theoretically, synaptic connections, knockout of SAP97 does not reliably prevent GluA1 surface expression (Kim et al., 2005; Zhou et al., 2008). Together, these findings indicate that SAP97 localization at the PSD and its interactions with proteins like GluA1-containing AMPARs and 4.1N, particularly in the context of cocaine priming-induced reinstatement, requires further investigation.

4.1N

4.1N is an accessory protein that contains an actin/spectrin binding domain and binds with the membrane proximal region of GluA1 subunits (Shen et al., 2000; Coleman et al., 2003; Anggono and Huganir, 2012). Phosphorylation of GluA1 at serines 816 and 818 by PKC regulates GluA1 association with 4.1N (Lin et al., 2009). Lin and colleagues (2009) have shown that interaction between GluA1 subunits and the actin/spectrin

binding protein 4.1N improves their extrasynaptic insertion. Accordingly, cocaine-mediated activation of PKC and subsequent GluA1 phosphorylation could contribute to the increased expression of GluA1 at the accumbens surface via 4.1N (Xue et al., 2012; Pierce and Wolf, 2013). Knock down of 4.1N or inhibition of PKC reduces insertion of native GluA1 into the plasma membrane, and mutant forms of GluA1 lacking the membrane proximal region show significantly reduced insertion (Lin et al., 2009). Further, 4.1N contains a FERM (Four.1-Ezrin-Radixin-Moesin) domain that promotes binding to the I_3 insert of the membrane-associated guanylate kinase, SAP97, to aid in SAP97 targeting to the membrane (Lue et al., 1994; McLaughlin et al., 2002; Hanada et al., 2003). These results suggest that 4.1N could be a novel target mediating the trafficking and stabilization of GluA1-containing AMPARs during cocaine reinstatement.

Viral Vector Methodologies

Given a growing body of evidence supporting the role of AMPA receptors and GluA1 subunit localization during cocaine priming-induced reinstatement, delineating the necessity of GluA1 and its associated proteins in a selective, time-specific manner is of utmost importance. Behavioral pharmacology offers manipulation of some receptors and related proteins at a particular point in time, such as during a reinstatement session, but specific drug interventions for all of the proteins mentioned thus far do not yet exist. With even the most specific compounds, the risk for off-target interactions and effects remains, particularly at concentrations required for functional consequences (Wang et al., 2012). Genetic manipulations in mice have become increasingly sophisticated; addiction researchers are able to directly target single amino acids in a particular protein (such as CaMKII), in a particular brain region (the striatum) and even in a particular timeframe (using inducible promoters) (Kourrich et al., 2012). Unfortunately, modeling cocaine self-administration and reinstatement in mice requires additional training time,

has a much higher attrition rate, and has greatly reduced behavioral output measures relative to paradigms using rats (Thomsen and Caine, 2007). While the same genetic manipulations in rats are in still their infancy, viral vector manipulations may provide a methodology that can tease apart the details of glutamatergic plasticity during cocaine priming-induced reinstatement (Simonato et al., 2000; Zheng et al., 2012). Further, the differing expression profiles of viruses, including latency, duration, and spread across neurons, allow researchers the opportunity to make temporally and spatially distinct manipulations of the reward system at various points throughout complex behaviors like those in the reinstatement paradigm.

Vector Options

Herpes simplex virus (HSV) has amplicon vectors with small plasmid size of 5-10kb but can hold a large cDNA insert of up to 150kb and can readily over-express the transgene of interest in high titers (over 10^8 infectious units/mL) (Jerusalinsky et al., 2012). Because HSV are replication-deficient, a 'helper virus' allows naturally neurotropic HSV to rapidly express the cDNA insert in neurons and glia, peaking 48-72 hours post-infection, without the risk of integrating into host chromosomes (Neve et al., 2005). This allows for time and location specific over-expression or knock down of a given protein with minimal toxicity to the animal and reduced probability of insertional mutagenesis. Further, the virus becomes dormant 7-10 days after the initial infection, allowing the host system involved to return to an endogenous state. Unfortunately, though HSV no longer contains infectious aspects of the herpes virus from which it is derived, there remains the risk of immune response and potential infection in those working with the vector (Braun, 2006).

HSV vectors are not the only option for expressing recombinant DNA *in vivo*. One alternative viral vector approach, adeno-associated virus (AAV), has a reduced biosafety risk but is much smaller with a limited construct size of 4.7kb (Coura and Nardi, 2007). While AAV has long lasting gene expression (on a scale of months), it is slow to initiate transgene expression due to the conversion of single-stranded AAV DNA to double stranded DNA in the host (Coura and Nardi, 2007). Further, the AAV viral genome inserts into chromosome 19, increasing the risk of unanticipated mutation due to potential disruption of a coding region of the genome (Kotin et al., 1990; Jerusalinsky et al., 2012). Another viral vector option is lentivirus, which can infect a variety of dividing and non-dividing CNS cells in addition to neurons and carries recombinant constructs almost twice the size of AAV (Davidson and Breakefield, 2003; Li et al., 2012). Similar to AAV, lentivirus produces long-lasting gene expression due to its ability to incorporate into the host genome, typically in non-coding regions of chromosomes (Sakuma et al., 2012). Unlike AAV, however, lentiviral vectors are derived from HIV, which dramatically increases the biosafety risk of using these vectors as recombination events could potentially produce replication-competent HIV (Sakuma et al., 2012).

In the case of modulating neuronal signaling during cocaine-priming induced reinstatement, HSV provides far more flexibility than AAV or lentivirus in terms of the type of constructs created, timing of expression, and duration of expression. Subsequently, experimenters can manipulate proteins at discrete periods during the paradigm (during reinstatement), without interfering with neurochemistry during other aspects of training (self-administration), and later confirm baseline behavioral and biochemical activity within the same animals (due to viral dormancy). In chapter three of this dissertation, HSV vectors are used to manipulate the molecular milieu of nucleus accumbens MSNs, specifically during cocaine priming-induced reinstatement. Vectors

that express wild-type or a dominant-negative mutant of GluA1 will be used to directly manipulate GluA1-containing AMPARs in an attempt to identify the necessity of CP-AMPARs during reinstatement. Additional vectors expressing a dominant-negative mutant of 4.1N or a micro RNA sequence that knocks down endogenous SAP97 expression will be employed to elucidate the trafficking, stabilization, and function of GluA1-containing AMPARs during cocaine priming-induced reinstatement.

The above outlines some of the profound cellular and molecular effects cocaine exposure has on the brain that mediate addiction and relapse. However, a growing body of evidence links cocaine exposure to modifications of inherited genetic material as well (Nestler, 2013). Indeed, cocaine use has spread into younger generations as evidence by the fact that 2% of American high school students, a demographic vulnerable to developing cocaine addiction, abuse the drug annually (Johnston et al., 2012; Wong et al., 2013). Investigating the mechanisms through which parental cocaine use influences the behaviors of subsequent generations are paramount to establish the appropriate interventions for cocaine addiction and relapse.

Epigenetic Inheritance and Drug Addiction

Certain evolutionary adaptations may occur via ancestral perturbations that disrupt the physiology and/or behavior of offspring (Feil and Fraga, 2012; Champagne, 2013). Exposure to famine in one generation correlates with an inability to appropriately process insulin two generations later (Pembrey et al., 2006). Prenatal exposure to bisphenol A, a chemical found in plastics that mimics sex hormones, has sex-specific influences on emotional regulation in children (Braun et al., 2011). Similarly, drug addiction in humans can often be tracked through family lines, with addictive behaviors passed down from parent to child, sometimes across multiple generations (Johnson and

Leff, 1999; Ivanov et al., 2012). Yet, the natural selection of "traditional" Darwinian evolution occurs over hundreds of years, begging the question: how could isolated challenges to one generation influence the DNA of subsequent generations? Epigenetics offers a mechanism through which gene transcription is regulated – not through direct changes to DNA base pairs, but via chemical modifications to DNA and its associated histones (Crews, 2008; Handel and Ramagopalan, 2010; Roth and Sweatt, 2011).

Epigenetic Modifications to DNA

In the cell nucleus, DNA is packaged as chromatin, which is made up of 147 base pair strands wrapped around proteins called histones (Nakao, 2001). In its most condensed state, chromatin is inactive and no gene transcription occurs, whereas transcriptional machinery readily accesses DNA when chromatin is in an open state (Nestler, 2013). Reversible modifications that alter the state of chromatin to increase or decrease transcription often include post-translational modifications of histone N-terminal tails, such as methylation, acetylation, and phosphorylation, or even methylation of DNA itself (Champagne, 2013). Transcriptionally inactive chromatin generally has methylated histones and unmethylated DNA, while transcriptionally active chromatin generally has acetylated histones and methylated DNA (Roth and Sweatt, 2011). The epigenome describes the genome-wide patterns of epigenetic modifications that increase or decrease gene transcription (Curley et al., 2011). It is likely that environmental challenges alter gene expression, potentially across generations, through epigenetic mechanisms like these.

Epigenetic Inheritance

Epigenetic modifications and correlated gene expression changes can be passed across generations in a context-dependent or germline-dependent manner (Crews, 2008). Instances where an environmental perturbation directly changes the methylation or acetylation of histones and DNA of the subject are considered context-dependent (Crews, 2008). Occasions where epigenetic modifications to DNA remain, independent of the initial cause and without need for repeated exposure, are considered germline-dependent (Crews, 2008; Curley et al., 2011). Transgenerational, epigenetic inheritance requires the transmittance of information about the epigenome through the germline (See Figure 1.5).

In utero or prenatal exposure to some insult, whether toxin or otherwise, offers one common example of context-dependent modifications (Crews, 2008; Champagne, 2013). Acute stress to pregnant mothers due to war or famine is correlated with increased incidence of schizophrenia in their children, presumably because these children were exposed to stress hormones *in utero* (St Clair et al., 2005; Malaspina et al., 2008). Similarly, gestational exposure to drugs of abuse, like nicotine or cocaine, is well established to have a negative impact on fetal and child development due to direct effects of the drugs on the fetal epigenome (Dow-Edwards, 2011; Abbott and Winzer-Serhan, 2012). Many preclinical studies identify impairments of executive function and memory, disrupted cortical and hippocampal development, and reduced motivation to respond for cocaine in offspring that were exposed to cocaine *in utero* (Hecht et al., 1998; Lidow, 2003; He et al., 2006b). As modeled in Figure 1.5, in such cases the fetus, including its developing germ cells, was directly exposed to the same environmental conditions as the mother, making any epigenetic modifications context-dependent.

Thus, the first point at which germline-dependent epigenetic inheritance can be identified is the third generation, as all prior progeny had some exposure to the original context (Curley et al., 2011; Champagne, 2013).

In studies of paternal transmission, information about experiences is limited to what is retained in the spermatozoa (Curley et al., 2011). There is evidence of children who display characteristics of fetal alcohol system with alcoholic fathers, but non-drinking mothers (Lemoine et al., 2003). The offspring of male rodents exposed to alcohol before mating with naïve females exhibit altered development of reflexes and disruption of metabolic functioning (Emanuele et al., 2001; Jamerson et al., 2004). The second generation (F_2) male offspring of prenatally stressed male mice exhibit brain dysmasculinization and a stress-sensitive behavioral phenotype (Morgan and Bale, 2011). In these scenarios, the third example of paternal transmission meets the requirements for germline-dependent epigenetic inheritance. The first two cases involve exposing sire sperm to alcohol, which means that half the genetic and epigenetic material of the first generation has also been exposed. In the third example, the F_2 generation has not experienced the stressful context of the first and originating generations, meaning any epigenetic changes are mediated via the germline, as modeled in Figure 1.5.

Epigenetic Transmission of a Cocaine Resistance Phenotype

Recently, our group established a paradigm using paternal (sire) cocaine self-administration to identify intergenerational consequences of cocaine abuse without directly exposing the fetus to cocaine (Vassoler et al., 2013). We identified behavioral and epigenetic evidence of a cocaine-resistance phenotype in first generation male offspring due to sire cocaine exposure. Male, but not female, cocaine-sired offspring

exhibited delayed acquisition and reduced maintenance of cocaine self-administration. Further, male offspring of cocaine-experienced sires displayed increased levels of brain derived neurotrophic factor (BDNF) in the mPFC, which has been previously reported to reduce the reinforcing efficacy of cocaine (Sadri-Vakili et al., 2010; Vassoler et al., 2013). Interestingly, spermatozoa contain binding sites for cocaine and decreased DNA methyltransferase 1, a key enzyme DNA methylation, is observed in sires that self-administered cocaine (Yazigi et al., 1991; Reik et al., 2001; He et al., 2006a). This model allowed for strict control of additional environmental influences on offspring, focusing on the effects of paternal exposure to cocaine on inherited genetic material. This may account for the discrepancy in our data with that of human literature identifying heritability of cocaine abuse (Kendler et al., 2007). That said, the presence of a cocaine *resistance* phenotype remains intriguing and has prompted additional exploration.

Anxiogenic Effects of Cocaine

Behavioral Consequences

Cocaine is well known for its euphoric effects, with addicts reporting feeling energetic and alert and having less need for food or sleep (Karch, 1999). However, those who abuse the drug indicate negative feelings of irritability and anxiety as early as 45 minutes after use, which can extend for days after the last exposure (Ward et al., 1997; Gunnarsdottir et al., 2000). Interestingly, individuals with anxious-impulsive traits are significantly more likely to develop cocaine dependence (Ersche et al., 2012).

These reports of cocaine-induced anxiety have been recapitulated in rodents. A 15 mg/kg cocaine injection (i.p.) produces anxiolytic effects 10 minutes after the injection yet anxiogenic effects 30 minutes following the injection (Muller et al., 2008). The competing positive and negative effects of cocaine, or opponent-processes, enroll

multiple brain systems and create ambivalence towards the drug despite chronic use (Ettenberg, 2004). In fact, the runway model of rodent self-administration, where rats are trained to run from a start box, down a runway, and into an infusion chamber, consistently captures an "approach-avoidance" behavior. Cocaine-addicted animals hesitate to enter, or even retreat from, the infusion box multiple times before finally entering to receive an infusion (Ettenberg, 2009). In this paradigm, the dorsal raphe, which produces the neurotransmitter serotonin and is involved in the expression of anxiety, can be reversible inactivated to reduce rodent avoidance behavior, preventing the anxiogenic effects of cocaine (Ettenberg et al., 2011).

In other studies, anxiogenic effects of cocaine include decreased self-administration (via lever press) and decreased levels of dopamine in the nucleus accumbens in continuously subordinate, but not episodically defeated, rats (Miczek et al., 2011). Rodents repeatedly injected with cocaine (20 mg/kg, i.p.) during adolescence display heightened anxiety-like responses to novelty in adulthood (Stansfield and Kirstein, 2007). Pharmacological agents used to reduce anxiety, like diazepam, enhance cocaine self-administration and reduce response latency without altering extracellular dopamine, suggesting correction of anxiety-like behavior without enhancement of reward (Maier et al., 2008). Rodents selectively bred for a depressive-like phenotype exhibit blunted mesocorticolimbic dopamine signaling in addition to reduced motivation to seek cocaine and attenuated cocaine priming-induced reinstatement (Lin et al., 2012).

Given these data, it is conceivable that the cocaine-resistance phenotype in male cocaine-sired offspring could be connected to an increased sensitivity to the anxiogenic effects of cocaine. Chapter four of this dissertation investigates the presence of an

anxiety phenotype in these offspring, particularly in conjunction with cocaine exposure, which could shed light onto additional mechanisms underlying the resistance to cocaine-seeking behavior. Of course, even the most robust behaviors must have cellular, molecular, and even genetic correlates. In addition to the aforementioned behavioral studies, there is a growing literature describing the neurological correlates between cocaine abuse and anxiety that may aid interpretations of these findings.

Neurological Correlates

Along with dopamine, cocaine prevents the reuptake of serotonin (5-HT), the binding of which to various 5-HT receptors located in the dorsal raphe contributes to anxiety and avoidance behaviors (Filip et al., 2005; Ettenberg et al., 2011; Donaldson et al., 2013). The mesocorticolimbic reward system processes the rewarding effects of cocaine but also shows involvement in cocaine-induced anxiety. During cocaine withdrawal, serotonin levels are decreased in the accumbens, the local reversal of which has been shown to alleviate withdrawal symptoms such as anxiety (Filip et al., 2005). Rats that display a characteristic anxiety-like behavior of excessive grooming take more cocaine and have reduced serotonin release relative to low grooming rats, though this difference is abolished with the administration of serotonergic anxiolytic agents (Homberg et al., 2004).

However, anxiety-like behaviors following cocaine exposure are not solely mediated via serotonergic transmission. The neuropeptide corticotropin releasing factor (CRF) and its two G-protein coupled receptors (CRF R1 and R2) have been illustrated to co-localize with dopamine terminals on neurons in the accumbens (Lemos et al., 2012). Activation of the CRF receptors has been illustrated to modulate HPA axis activation and anxiety-like behavior (Bale and Vale, 2004). Lemos and colleagues (2012) illustrated

acute activation of accumbal CRF receptors promotes dopamine release and CPP whereas chronic activation, traditionally correlated with anxiety phenotypes, does not. In rats, BDNF exhibits a time-dependent increase in the accumbens during the incubation of cocaine craving which correlates to a period where human addicts describe strong feelings of agitation and anxiousness (Grimm et al., 2003). One study found repeated experimenter-administered cocaine increases signaling of accumbens BDNF and rodent susceptibility to social defeat via epigenetic modifications of chromatin like histone methylation and downregulation of methyltransferases (Covington et al., 2011).

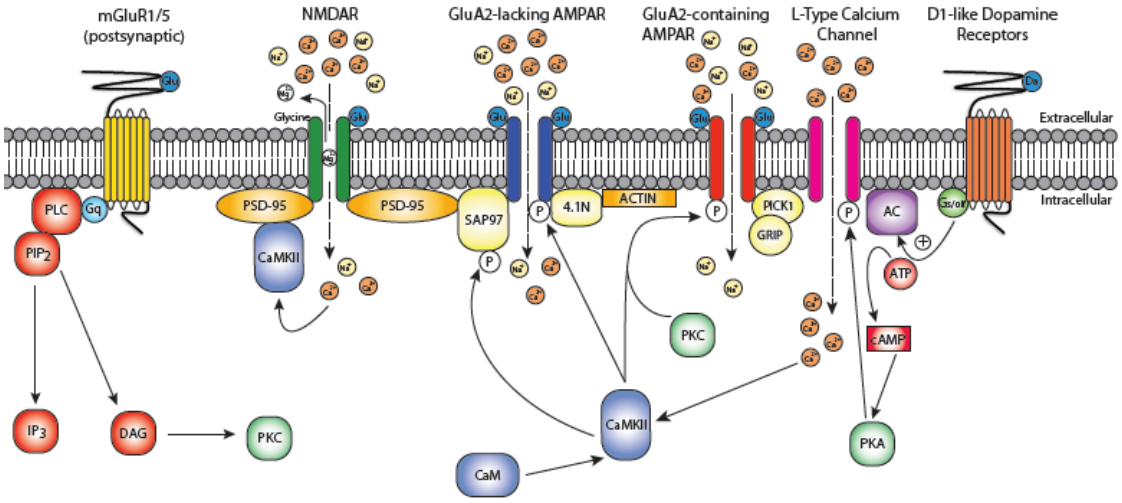
Another limbic region involved in the expression of anxiety and influenced by cocaine exposure is the hippocampus (Deschaux et al., 2012; Kheirbek et al., 2013). The hippocampus contains receptors for the corticosterone and CRF released following HPA axis activation, and sends negative feedback projections to deactivate the stress system (Herman et al., 2005). Additionally, serotonergic transmission in the hippocampus is reduced by exposure to psychostimulants, potentially via down-regulation of a major corticosteroid stress receptor (Barr and Forster, 2011). Prenatal stress increases anxiety-like behaviors in offspring as well as levels of hippocampal proBDNF, the BDNF precursor that activates cell death pathways and increases long term depression in hippocampal synapses (Martinowich et al., 2007; Yeh et al., 2012). Altered BDNF levels in the hippocampus are associated with decreased anxiety-like behavior in the novelty induced hypophagia task, and serontonergic transmission has been shown to underlie changes in BDNF mRNA expression in stressed animals (Vaidya et al., 1999; Schmidt and Duman, 2010). Additionally, withdrawal from repeated experimenter-delivered cocaine enhances hippocampal LTD, an effect that is blocked by application of a CRF R2 antagonist. Finally, prenatal cocaine exposure modulates

hippocampal DNA methylation in newborn and adolescent rodents, altering the expression of a variety of genes (Novikova et al., 2008; Guan et al., 2009).

Together, these experiments indicate that limbic system modulators of the stress and anxiety pathway, such as serotonergic, BDNF, and CRF signaling, are likely to mediate anxiety-like behaviors following cocaine exposure. In addition to behavioral characterization, chapter four of this dissertation examines the consequences on stress and anxiety systems due to paternal cocaine experience by delineating the neurochemical phenotypes of cocaine-sired offspring. Experiments in this chapter will examine cellular and molecular correlates of anxiety, such as corticosterone, serotonin, BDNF, and CRF expression, in drug naïve cocaine- and saline-sired male offspring to identify changes due solely to paternal cocaine exposure.

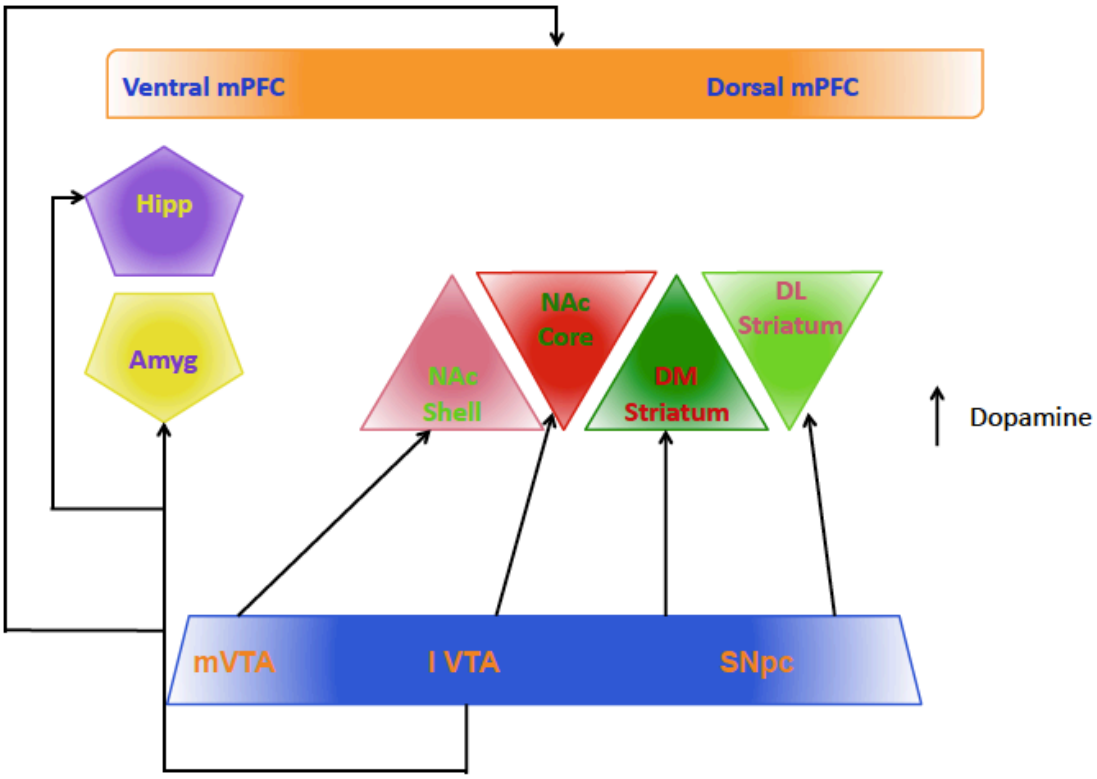
Exposure to cocaine has myriad consequences, extending from immediate biochemical adaptations in the brain to neurological and behavioral changes in subsequent generations. Focusing on cocaine-induced changes to the glutamatergic system, chapters two and three use a rodent model of cocaine self-administration and reinstatement to examine select brain regions and receptors involved during initial use (chapter two) and during priming-induced reinstatement (chapter three). Subsequently, chapter four expands the scope of this dissertation beyond the effects of cocaine on the user, to include the influence of paternal cocaine experience on first generation behavior and neurochemistry. As a whole, the work encompassed in this dissertation advances our understanding of both the biochemical mechanisms underlying and the heritable consequences of cocaine use and relapse.

FIGURE 1.1



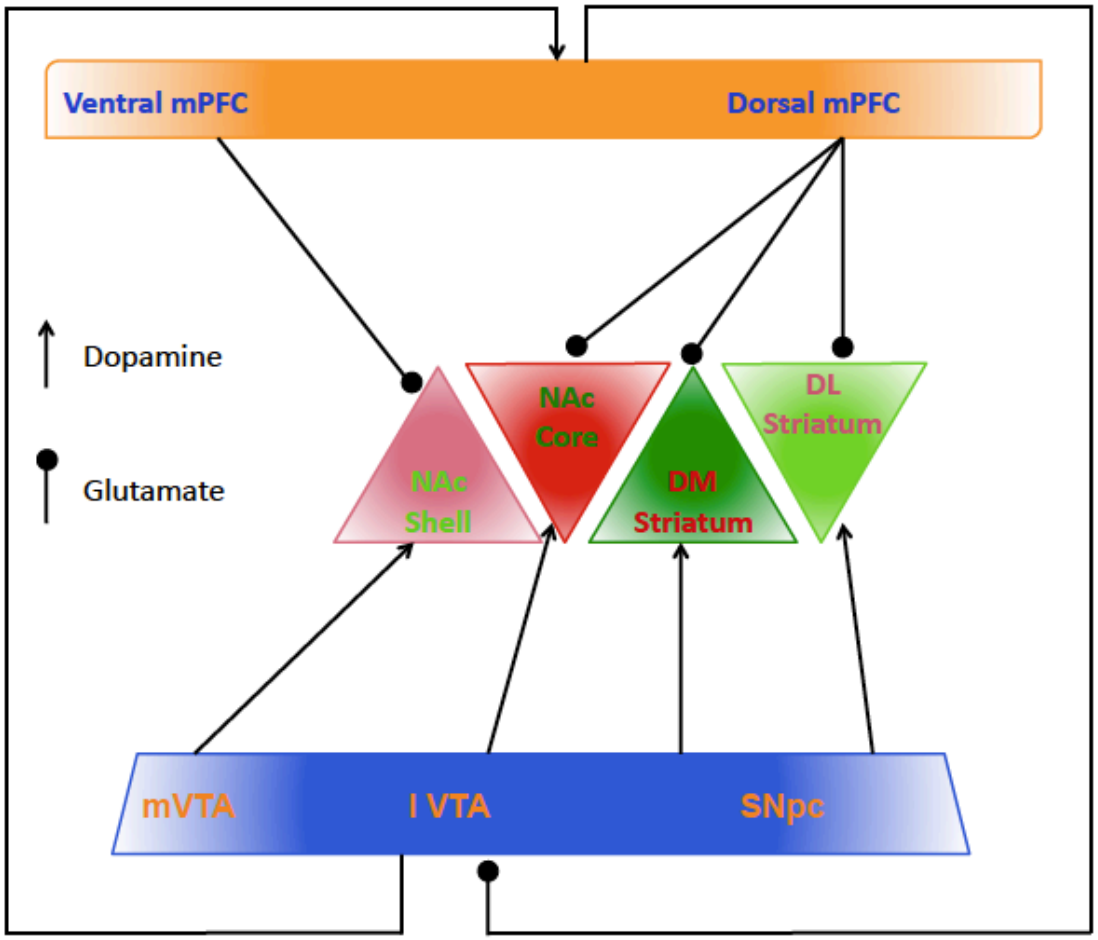
Glutamate receptor–mediated signaling. Glutamate released into the synaptic cleft binds to and activates ionotropic glutamate receptors (NMDA, AMPA, and kainate [KA] receptors) on postsynaptic membranes. Due to enzymatic editing of GluA2 AMPAR subunits, GluA2-containing AMPARs are calcium impermeable, while GluA2-lacking AMPARs are calcium permeable. Extracellular glutamate also binds to and activates perisynaptic metabotropic glutamate receptors located on postsynaptic (mGluR1/5s heteroreceptors) membranes. Influx of Na+, Ca2+, and K+ ions through activated AMPA/KA receptors depolarizes the neuron and subsequently relieves the Mg2+ block from voltage-sensitive NMDA receptors and activates L-type voltage-gated Ca2+ channels. In addition to propagating action potentials, influx of cations through ionotropic glutamate receptors activates several intracellular signaling pathways including, but not limited to, PKC, CaMKII, and protein kinase A (PKA). These kinases phosphorylate various residues in the ionotropic glutamate receptors to modulate their channel properties. Group I (mGluR1/5) metabotropic glutamate receptors are coupled via Gq to intracellular enzymes. Stimulation of mGluR1/5s activates phospholipase C (PLC), which catalyzes the production of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol-4,5 bisphosphate (PIPs). The resulting increase in cytoplasmic IP3 triggers release of Ca2+ from intracellular stores (not shown). Also present are metabotropic D1-like (D1, D5) dopamine receptors that initiate intracellular signaling cascades via Gs and Golf. Stimulation of D1-like receptors promotes adenylyl cyclase (AC) activity, thus increasing intracellular levels of cAMP and PKA. The cytoplasmic proteins PSD-95, glutamate receptor-interacting protein (GRIP), synapse associated protein 97 (SAP97), protein 4.1N, and actin anchor GluA2- and GluA1-containing glutamate receptors to the PSD complex. Cocaine exposure induces phosphorylation events by CaMKII, PKC, and/or PKA that modulate interactions between receptor subunits and these proteins to alter the population of AMPARs at the synapse, changing the excitability of the post-synaptic cell. (Figure adapted from: Schmidt and Pierce, 2010).

FIGURE 1.2



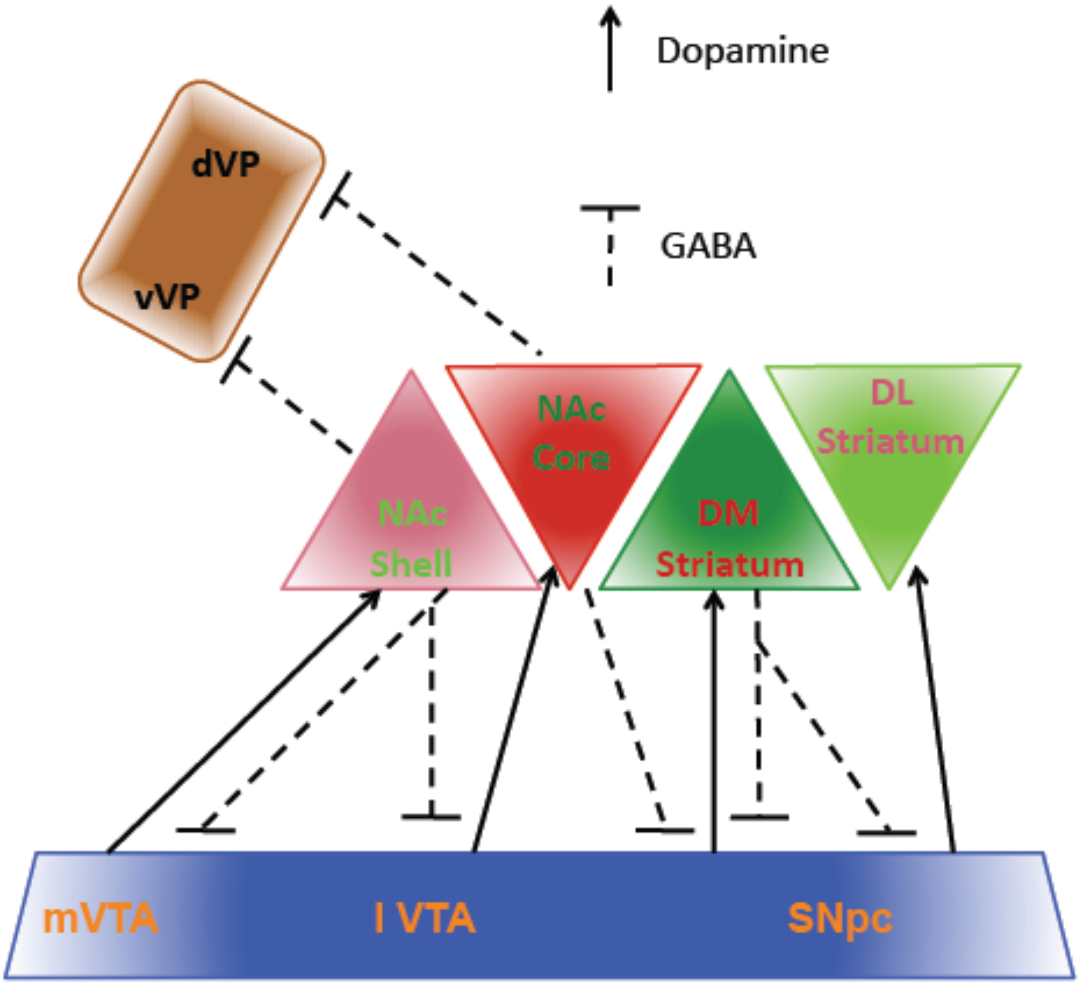
Dopaminergic projections through the mesocorticolimbic reward system. Schematic of dopaminergic projections from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) enlisted during the processing of rewards. Cocaine priming-induced reinstatement of drug seeking modulates the release of dopamine across the striatum in the subregions of the nucleus accumbens (NAc) and the dorsomedial (DM) and dorsolateral (DL) striatum. Dopaminergic signaling of cocaine reward also occurs in limbic structures such as the hippocampus (Hipp) and amygdala (Amyg), and areas of executive function like the medial prefrontal cortex (mPFC).

FIGURE 1.3



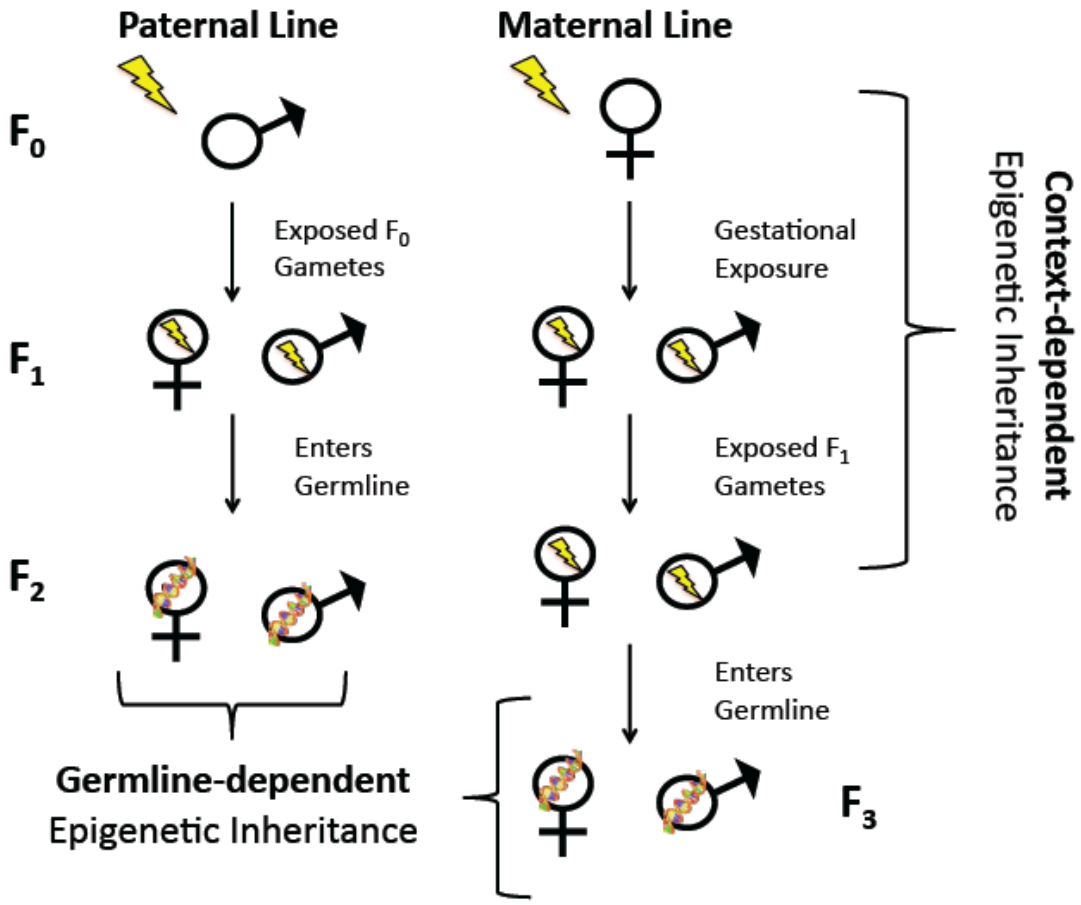
Glutamatergic projections modulate processing in the mesocorticolimbic reward system. Schematic of the dopaminergic and glutamatergic projections in the mesocorticolimbic reward circuitry. Cocaine exposure directly influences dopaminergic projections initiating from midbrain dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) that terminate in the nucleus accumbens (NAc), dorsomedial (DM) and dorsolateral (DL) striatum, and medial prefrontal cortex (mPFC). Cocaine exposure indirectly influences glutamatergic projections initiating in the mPFC that provide excitatory feedback to the VTA in addition to modulating the excitation of neurons across the striatum.

FIGURE 1.4



Progressive recruitment of striatal subregions as cocaine use becomes habitual. Schematic of the dopaminergic and GABAergic projections in midbrain and striatal reward circuitry. Initial exposure to cocaine activates dopaminergic projections from the medial ventral tegmental area (mVTA) to the nucleus accumbens (NAc) shell, which then provides GABAergic inhibitory feedback across the VTA, modulating subsequent dopaminergic projections to the NAc core. With continued cocaine experience, the core sends GABAergic projections to the substantia nigra pars compacta (SNpc), which modulates dopaminergic projections from the SNpc to the dorsomedial (DM), followed by the dorsolateral (DL) striatum. The shell and core also send GABAergic projections to the ventral and dorsal ventral pallidum (vVP, dVP) that modulates limbic and motor processing, respectively.

FIGURE 1.5



Parental environmental perturbations can produce context- and germline-dependent epigenetic modifications to genetic material transmitted across generations. Schematic of how epigenetic modifications due to an environmental challenge (lightening bolt) such as cocaine exposure in one generation transmits to subsequent generations. In a maternal lineage, epigenetic modifications are context-dependent through the first two generations (F_1 and F_2) as the gametes of the developing fetus are exposed to the same environmental perturbations as the mother. By the third generation (F_3), remaining epigenetic modifications have entered the germline. In a paternal lineage, epigenetic modifications switch from context- to germline-dependent by the second generation as only the gametes of the father are exposed to the environmental challenge.

CHAPTER TWO

ACUTE COCAINE INCREASES PHOSPHORYLATION OF CAMKII AND GLUA1 IN THE DORSOLATERAL STRIATUM OF DRUG NAÏVE RATS, BUT NOT COCAINE-EXPERIENCED RATS.

Samantha L. White[1], Heath D. Schmidt[1], Fair M. Vassoler[2], R. Christopher Pierce[1]

1: Center for Neurobiology and Behavior

Department of Psychiatry

Perelman School of Medicine

University of Pennsylvania

125 South 31st Street, Rm 1102a

Philadelphia, PA, USA 19104

2: Department of Biomedical Sciences

Tufts University, 200 Westboro Rd

North Grafton, MA, USA 01536

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Abstract

Transport of GluA1-containing AMPA glutamate receptors to synapses in the nucleus accumbens, a process that involves phosphorylation of key serine residues by CaMKII, is associated with the reinstatement of cocaine-seeking behavior. A growing body of evidence indicates that the dorsal striatum contributes to aspects of cocaine addiction. However, the potential role of CaMKII-mediated phosphorylation of GluA1 subunits in the dorsolateral (DL) striatum during cocaine reinstatement has not been examined. In this study, rats were trained to self-administer cocaine and were partnered with saline-yoked rats that received injections of saline. Following extinction, each pair of rats received either a systemic priming injection of cocaine (10 mg/kg, i.p.) or saline. As expected, cocaine-experienced rats displayed robust reinstatement of cocaine seeking in response to a challenge injection, whereas yoked saline controls did not. The DL striatum was dissected immediately following the reinstatement test session. Results from Western blotting experiments showed increased pGluA1-ser831 and pCaMKII-thr286 in the DL striatum of saline-yoked rats given an acute injection of cocaine. This effect was absent in cocaine-experienced rats that received a saline injection, and no changes were observed following a priming injection of cocaine in cocaine-experienced rats. These results indicate that acute exposure to cocaine in drug naïve rats increased CaMKII-mediated phosphorylation of GluA1-containing AMPA receptors in the DL striatum, an effect that was not observed during cocaine priming-induced reinstatement of drug seeking. It is possible, therefore, that increased phosphorylation of CaMKII and GluA1 following acute cocaine is a compensatory mechanism in the DL striatum.

Introduction

It is well documented that changes in glutamatergic transmission in subregions of the striatum play a role in the reinstatement of cocaine seeking, an animal model of relapse (Pierce and Vanderschuren, 2010). Although the majority of this work has focused on the nucleus accumbens (Anderson et al., 2008; Famous et al., 2008; Schmidt and Pierce, 2010), recent evidence indicates that pharmacological inhibition of the dorsolateral (DL) striatum attenuates cocaine seeking in rats following short-access (1- or 2-hour daily) self-administration (See et al., 2007; Pacchioni et al., 2011). In terms of glutamatergic transmission, infusion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) glutamate receptor antagonists into the DL striatum blocked cue-controlled reinstatement and attenuated cocaine self-administration when injected into the dorsomedial (DM) striatum (Vanderschuren and Everitt, 2004; Famous et al., 2008; Murray et al., 2012; Schneck and Vezina, 2012). Though there is an established role for the DL striatum in cue-induced reinstatement, there is little to no data on DL striatum involvement in cocaine priming-induced reinstatement of drug seeking.

AMPA glutamate receptors are ionotropic, excitatory ion channels comprised of four subunits (GluA1-GluA4) that are involved in several forms of neuronal plasticity (Bredt and Nicoll, 2003). The GluA1 subunit in particular has been associated with synaptic plasticity in animals exposed to cocaine (Boudreau and Wolf, 2005; Bachtell et al., 2008). Activity-mediated autophosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII) at threonine 286 promotes phosphorylation of GluA1 at serine 831, resulting in enhanced GluA1 subunit trafficking to the plasma membrane and associated increases in synaptic strength (Lisman et al., 2002; Bredt and Nicoll, 2003). Indeed, there is evidence that reinstatement of cocaine seeking in rats involves CaMKII phosphorylation of the GluA1 subunit in the nucleus accumbens, and pharmacological

inhibition of CaMKII in the accumbens prevents cocaine seeking (Mammen et al., 1997; Anderson et al., 2008).

A single cocaine injection promotes glutamate release in the DL striatum of cocaine-naïve rats (Barrot et al., 2001; McKee and Meshul, 2005). Acute or repeated experimenter-delivered cocaine increases levels of phosphorylated GluA1 AMPA receptor subunits and GluA1 surface expression in the DL striatum (Kim et al., 2009b; Ferrario et al., 2010). Here, we extend this work by examining expression of phospho-CaMKII and –GluA1 in the DL striatum during cocaine priming-induced reinstatement of drug seeking.

Materials and Methods

Animals and Housing

Male Sprague-Dawley rats (*Rattus novergicus*) weighing 250-300 g were obtained from Taconic Laboratories (Germantown, NY, USA). Animals were individually housed with food and water available *ad libitum*. A 12h light/dark cycle was used and all experiments were performed during the light cycle. All experiments used Med-Associates (East Fairfield, VT, USA) operant chambers equipped with response levers, house light, and pumps for injecting drugs intravenously. Operant chambers were enclosed within ventilated, sound attenuating chambers. All experimental procedures were consistent with the ethical guidelines of the U.S. National Institutes of Health and were approved by the University of Pennsylvania School of Medicine Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Rockville, MD, USA) and dissolved in bacteriostatic 0.9% saline.

Surgery

Prior to surgery, the rats were anesthetized with 80 mg/kg ketamine (i.p.; Sigma-Aldrich, St. Louis, MO, USA) and 12 mg/kg xylazine (i.p.; Sigma-Aldrich, St. Louis, MO, USA). An indwelling silastic catheter (CamCaths, UK) was placed into the right jugular vein and sutured in place. The catheter was routed to a mesh backmount platform and implanted subcutaneously dorsal to the shoulder blades. Catheters were flushed daily with 0.3 ml of an antibiotic (Timentin, 0.93 mg/ml, Henry Schein, Melville, NY, USA) dissolved in heparinized saline. The catheters were sealed with plastic obturators when not in use.

Cocaine Self-Administration and Extinction

Following a 7-day recovery period from surgery, 50% of the rats were randomly selected to self-administer cocaine. The remaining 50% were paired to a cocaine self-administering animal as yoked-saline controls that only receive infusions of saline. Cocaine self-administering rats were placed in operant chambers and allowed to lever press for intravenous cocaine infusions (0.25 mg/59 μl saline over 5 s) over a 2-hour time period daily for 16 days. Yoked-saline controls received the same number and temporal pattern of infusions as the paired cocaine self-administering rat. Rats were initially trained using a fixed ratio (FR) 1 schedule of reinforcement, with each daily self-administration session initiated by a priming injection of cocaine (0.25 mg, i.v.) to fill the catheter (limited to 30 injections per 120 min). Once animals achieved stable responding, as defined by at least 20 infusions over the 2 h session for 2 consecutive days, they were transitioned to an FR5 schedule of reinforcement (limited to 30 injections per 120 min). For both schedules of reinforcement, a 20s time-out period followed each cocaine infusion, during which time active, drug-paired lever responses were tabulated but had no scheduled consequence. Each operant chamber was also equipped with an inactive lever. Responses made on the inactive lever, which had no scheduled consequence, were also recorded during all training sessions.

Following cocaine self-administration, drug-taking behavior in cocaine self-administering rats was extinguished by replacing cocaine with 0.9% bacteriostatic saline. Extinction continued until responding on the drug-paired lever was <15% of the response rate on the last day of cocaine self-administration using the FR5 schedule of reinforcement. Typically, it took rats 5 days to meet this criterion.

Cocaine Priming-Induced Reinstatement of Drug Seeking

Following extinction, animals entered the reinstatement phase of the experiment. During reinstatement, satisfaction of the response requirements for FR5 resulted in saline rather than cocaine infusions. The FR5 schedule was used for the reinstatement test session. Pairs of cocaine self-administering/yoked-saline rats were randomly assigned to receive saline or cocaine (10 mg/kg, i.p.) injections immediately prior to the start of the reinstatement test session. Rats were placed into the operant chambers immediately following injection of saline or cocaine. Responding was recorded for 30 minutes, after which the pairs of rats were removed from the operant chambers and immediately decapitated. Whole brains were extracted and flash-frozen in isopentane on dry ice, then stored at -80° C. Brains were sliced on a cryostat (300 μm sections) and striatal subregions (+1.7 mm to –0.08 mm A/P; –3.8 mm to –6.0mm D/V; DM: ±1.2 mm – 2.8 mm M/L and DL: ±2.8 mm – 4.4 mm M/L) dissected by tissue punch (2.0 mm Harris Uni-core stainless steel punchers, Ted Pella, Inc.). Tissue samples were stored at -80° C until processing for Western blot as described below.

Western Blot

Whole-cell tissue was processed for Western blot as described previously (Anderson *et al*., 2008). For all samples, protein concentration was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (10 μg for whole-cell) were loaded and separated in 10% Tris-Glycine gels (Invitrogen) and transferred to nitrocellulose membranes using the i-Blot dry transfer system (Invitrogen). Membranes were blocked with either 5% non-fat dry milk in TBST or 5% BSA in TBST, according to antibody instructions. Membranes were incubated overnight at 4° C with selective antibodies to: GluA1 (1:1000, Abcam), phosphorylated (p) GluA1-S831 (1:500,

Millipore), CaMKII (1:1000, Millipore), pCaMKII-T286 (1:1000, Cell Signaling), and GAPDH (1:2000, Cell Signaling). Membranes were then incubated with fluorescent secondary antibodies (1:5000, IR-dye 680 or IR-dye 800), before being imaged on an Odyssey fluorescent scanner (Licor Biosciences). To ensure equal loading, GAPDH expression was used as a loading control.

Behavioral Experiment Data Analyses

Drug-paired and inactive lever responding was analyzed using a two-way ANOVA. The between subjects factors were pretreatment (cocaine self-administration or yoked-saline) and drug challenge injection (10 mg/kg cocaine or saline). Bonferroni post-tests were used to establish significant difference ($p<0.05$).

Western Blot Data Analyses

Quantification was performed by normalizing the intensity of all bands with protein-specific antibodies to the GAPDH intensity, followed by normalizing that value to saline-control (yoked-saline, i.p. saline) values. The immunoblot analyses were performed using a two-way ANOVA. The between-subjects factors were pretreatment (cocaine self-administration or yoked-saline) and drug challenge injection (10 mg/kg cocaine i.p. or saline). Bonferroni post-tests were used to establish significant difference ($p<0.05$).

Results

A priming injection of cocaine, but not saline, elicits robust reinstatement of drug-seeking behavior in animals with cocaine self-administration experience.

Total drug-paired lever responding (mean +/- SEM) for the reinstatement test session are plotted in Fig 2.1. Responding was analyzed using a two-way ANOVA. Analyses revealed a significant main effect of pretreatment ($F_{1,24}$=119.39, p<0.0001), a significant main effect of challenge injection ($F_{1,24}$=92.14, p<0.0001), as well as a significant pretreatment × challenge interaction ($F_{1,24}$=78.93, p<0.0001). *Post-hoc* tests revealed a significant difference of drug-paired lever responding due to cocaine challenge injection (Bonferroni p<0.001). There was no significant effect of pretreatment ($F_{1,24}$=0.03, p=0.8543) or challenge ($F_{1,24}$=3.68, p=0.0671) on inactive lever responding (data not shown).

Exposure to acute cocaine in drug naïve animals increases expression of pGluA1-ser831 and pCaMKII-thr286 in the DL Striatum.

The average fluorescent intensity for pGluA1-ser831 in the DL striatum was expressed as percent change from control and is plotted in Fig 2.2A. Percentages were analyzed by two-way ANOVA, which revealed a significant main effect of pretreatment (cocaine self-administration vs. yoked-saline, $F_{1,12}$=14.03, p<0.005), a significant main effect of challenge (10 mg/kg cocaine or saline, $F_{1,12}$=30.68, p<0.0001), as well as a significant pretreatment × challenge interaction ($F_{1,12}$=20.75, p<0.0007). *Post hoc* tests revealed a significant difference in pGluA1-ser831 expression between yoked-saline and cocaine self-administering animals after cocaine challenge injection (Bonferroni p<0.001). There was no significant effect of pretreatment or challenge on the native GluA1 protein (Fig 2.2B).

The average fluorescent intensity for pCaMKII-thr286 in the DL striatum was expressed as percent change from control and plotted in Fig 2.2C. Percentages were analyzed by two-way ANOVA, which revealed a significant main effect of pretreatment ($F_{1,11}$=15.73, p<0.0022), a significant main effect of challenge ($F_{1,11}$=48.62, p<0.0001), as well as a significant pretreatment × challenge interaction ($F_{1,11}$=9.78, p<0.0096). *Post hoc* tests revealed a significant difference in pCaMKII-thr286 expression between yoked-saline and cocaine self-administering animals after cocaine challenge injection (Bonferroni p<0.01). There was no significant effect of pretreatment or challenge on native CaMKII protein (Fig 2.2D).

Acute cocaine decreases expression of pGluA1-ser831, but not pCaMKII-thr286, in the DM striatum regardless of cocaine exposure.

The average fluorescent intensity for pGluA1-ser831 in the DM striatum was expressed as percent change from control and plotted in Fig 2.3A. Percentages were analyzed with a two-way ANOVA, which revealed no significant effect of pretreatment (p=0.3542) and no pretreatment × challenge interaction (p=0.9403), but a significant main effect of challenge ($F_{1,24}$=4.33, p<0.05). There was no significant effect of pretreatment or challenge on the average fluorescent intensity of native GluA1 protein in the DM striatum (Fig 2.3B).

The average fluorescent intensity for pCaMKII-thr286 in the DM striatum was expressed as percent change from control and is plotted in Fig 2.3C. Percentages were analyzed with a two-way ANOVA, which revealed no significant effect of pretreatment (p=0.1615) or challenge (p=0.2906). There was no significant effect of pretreatment or challenge on the average fluorescent intensity of native CaMKII protein in the DM striatum (Fig 2.3D).

Discussion

The present findings indicate that an acute injection of cocaine increased expression of pCaMKII-thr286 and pGluA1-ser831 in the DL, but not the DM, striatum of drug-naïve rats. Since the phosphorylation of CaMKII at threonine 286 is associated with increased phosphorylation of GluA1 at serine 831 and subsequent trafficking of these subunits (Boehm and Malinow, 2005), these results suggest that an acute cocaine injection may increase the surface expression of AMPA receptors in the DL striatum. However, following cocaine self-administration and extinction, a cocaine challenge injection had no effect on DL striatal pCaMKII-thr286 or pGluA1-ser831 expression. Taken together, these results indicated that tolerance to cocaine-induced increases in the phosphorylation of DL striatal CaMKII and GluA1 develops following cocaine self-administration.

Our findings are consistent with previous work that showed increased pCaMKII-thr286 and pGluA1-ser831 in the DL striatum following acute cocaine (Edwards et al., 2007; Kim et al., 2009a). A single exposure to cocaine also increases immediate early gene expression in the dorsal striatum (Graybiel et al., 1990; Larson et al., 2010; Philibin et al., 2011). Additionally, a single microinjection of cocaine into the dorsal striatum enhances locomotor activity (Delfs et al., 1990). Taken together, these results indicate that cocaine-induced hyperlocomotion is associated with increased neuronal activation in the DL striatum and increased phosphorylation of CaMKII and GluA1. Future studies are required to determine the functional significance of increased phosphorylation of CaMKII and GluA1 following acute cocaine exposure.

There also is evidence of altered glutamatergic transmission in the DL striatum following prolonged cocaine administration (Porrino et al., 2007). Animals identified as

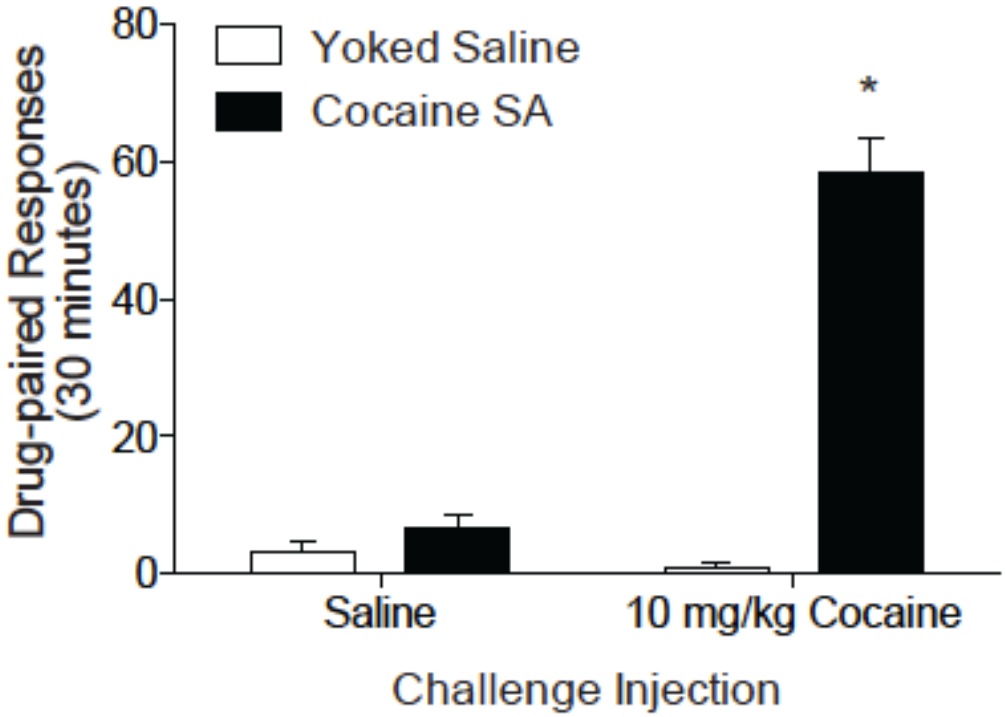
vulnerable to cocaine relapse following cocaine self-administration showed down-regulation of genes for GluA1 and activity-regulated cytoskeletal protein, both of which are involved in synaptic plasticity and AMPA receptor trafficking (Brown et al., 2011). While we did not investigate alternate phosphorylation sites of GluA1 subunits in this study, there is also evidence of decreased expression of phospho-GluA1 ser845, a known target of protein kinase A, in the dorsal striatum in animals with chronic cocaine self-administration experience (Mattson et al., 2005; Edwards et al., 2007). Functionally, pharmacological inactivation of the DL striatum attenuates cocaine seeking in rats with a history of short- or long-access to cocaine self-administration (Pacchioni et al., 2011). Moreover, an intra-striatal infusion of an AMPA/kainate receptor antagonist attenuates cue-controlled cocaine seeking (Vanderschuren et al., 2005; Pacchioni et al., 2011). Furthermore, overexpression of a dominant negative peptide that prevents trafficking of GluA1 to the membrane surface in the DL striatum attenuated cocaine-induced behavioral sensitization in juvenile mice (Kim et al., 2009a). It is important to note, however, that unlike adult mice, the juveniles exhibited increased GluA1 surface expression following repeated cocaine, suggesting that age at time of cocaine exposure influences DL striatal glutamatergic plasticity. Transgenic mice that express constitutively active CaMKII, where threonine 286 is replaced with aspartic acid, in the dorsal striatum exhibit impairments in goal-directed behaviors, with impairments in both cue- and reward-primed reinstatement (Wiltgen et al., 2007). As a whole, these results indicate that the changes in AMPA receptor transmission in adult rodents that contribute to cocaine sensitization and reinstatement are not likely due to increases in CaMKII or GluA1 phosphorylation in the DL striatum. Our data showed changes the phosphorylation state of CaMKII and GluA1 in DL striatum of drug naïve rats following acute cocaine only, offering novel biochemical support for such conclusions.

The present findings suggest differential involvement of the glutamatergic system in the DL striatum relative to the ventral striatum, particularly after cocaine self-administration and the reinstatement of cocaine seeking. It is well known that CaMKII plays a key role in phosphorylating GluA1 subunits at serine 831 and promotes AMPA receptor trafficking to the membrane surface (Boehm and Malinow, 2005). In fact, plasticity of the glutamatergic system in the ventral striatum, including increased phosphorylation of CaMKII and GluA1 as well as increased GluA1 surface expression occurs in a variety of paradigms involving chronic cocaine exposure (Boudreau and Wolf, 2005; Conrad et al., 2008; Ferrario et al., 2011). More specifically, during cocaine priming-induced reinstatement, there is increased phosphorylation of CaMKII and GluA1 AMPA receptor subunits, as well as increased trafficking of GluA1 to the plasma membrane in the accumbens shell (Anderson et al., 2008). Inhibiting CaMKII or preventing the trafficking of GluA1 subunits in the shell is sufficient to block reinstatement of cocaine seeking (Anderson et al., 2008). These findings coupled with the data presented here suggest that phosphorylation of CaMKII and GluA1 in the ventral, but not DL, striatum is required for reinstatement of cocaine seeking. Thus, changes in phospho-CaMKII and –GluA1 in the DL striatum likely reflect a compensatory mechanism in response to acute cocaine.

Our results indicate that acute cocaine increases expression of pCaMKII-thr286 and pGluA1-ser831 in the DL, but not DM, striatum and that these effects are not associated with the reinstatement of cocaine seeking. Therefore, changes in phospho-CaMKII and –GluA1 most likely reflect a compensatory adaptation in the DL striatum during early stages of drug use. It is possible that acute cocaine promotes transient biochemical modifications in the DL striatum that reverse with additional input from areas

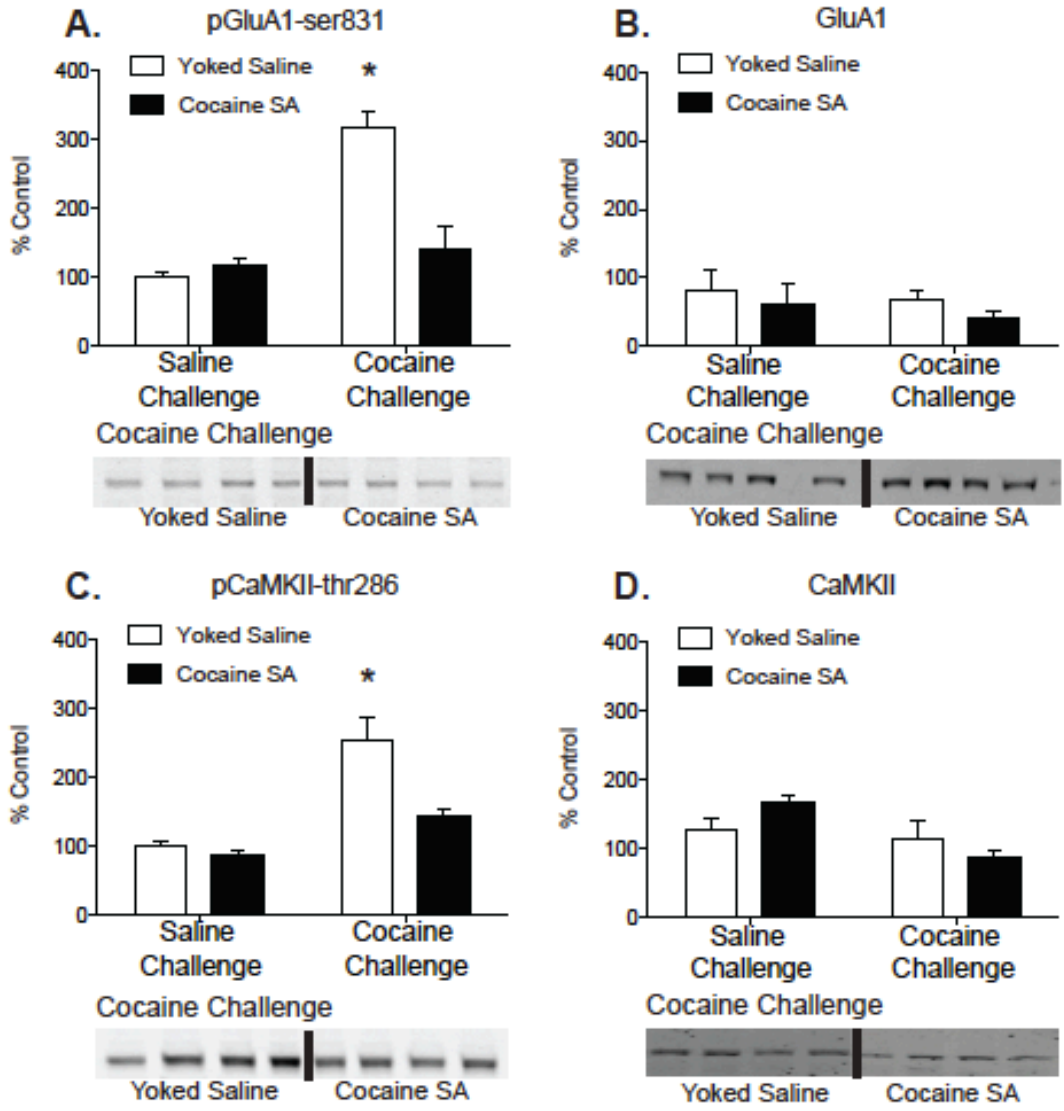
like the ventral striatum and/or prefrontal cortex as cocaine experience is extended (Edwards et al., 2007).

FIGURE 2.1



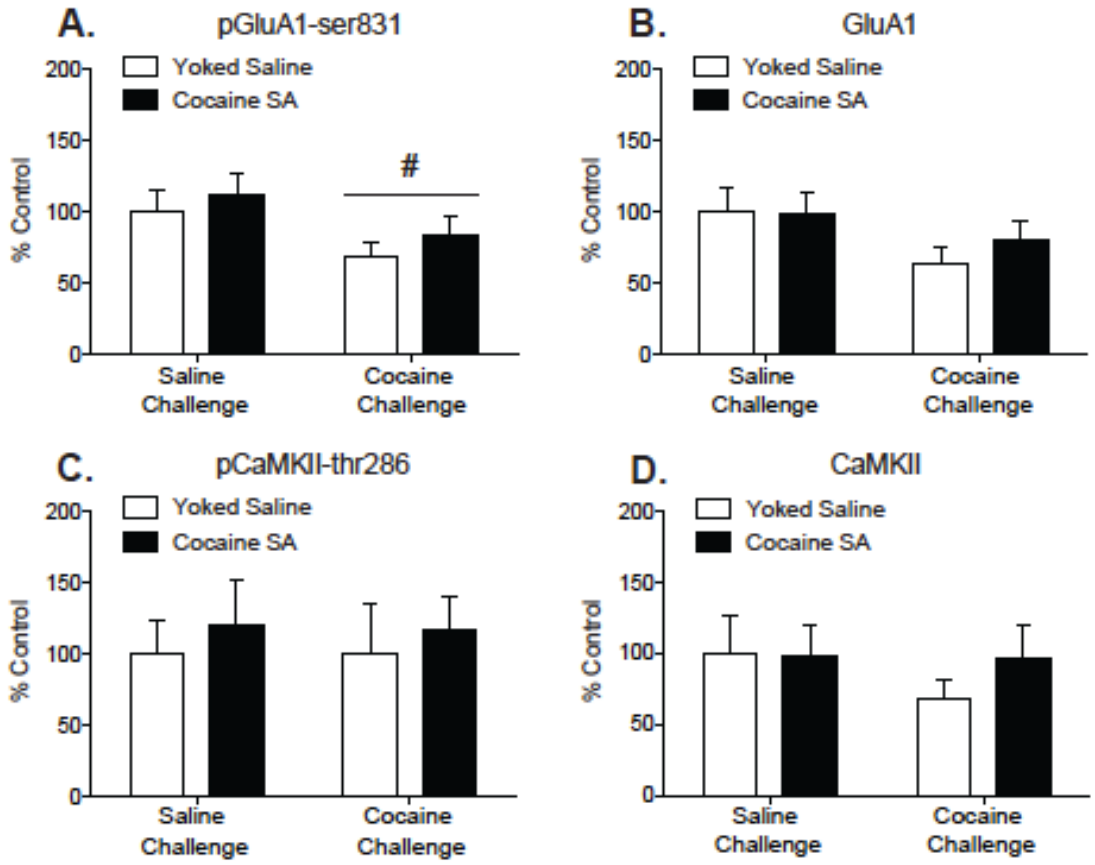
An acute, priming injection of cocaine elicits robust reinstatement of drug-seeking behavior in rats with cocaine self-administration experience. Total drug-paired lever responding for the 30-minute reinstatement test session is plotted for animals that received saline or cocaine challenge injection. There was a significant increase of drug-paired lever responding for cocaine-experienced rats that received cocaine compared to animals that received a saline injection. N = 7/group, "SA" = self-administration (The asterisk represents a significant difference from yoked-saline controls. 2-way ANOVA: Bonferroni $p<0.001$)

FIGURE 2.2



Acute cocaine exposure increases expression of pGluA1-ser831 and pCaMKII-thr286 in the DL striatum and these effects are reversed after cocaine self-administration. Following an acute cocaine injection, yoked-saline controls show a significant increase in pGluA1-ser831 (A) and pCaMKII-thr286 (B) protein expression in the DL striatum. No significant changes in pGluA1-ser831 (A) and pCaMKII-thr286 (B) protein expression were observed in cocaine-experienced animals when compared to yoked-saline controls that received saline injections. There was no significant effect of training or challenge injection on native GluA1 (C) or native CaMKII (D) protein expression for any treatment. N = 4-7/group, “SA” = self-adminstration (Asterisks represent significant differences from yoked-saline controls that received a saline challenge injection. 2-way ANOVA: pGluA1 Bonferroni $p<0.001$; pCaMKII Bonferroni $p<0.01$)

FIGURE 2.3



An acute, priming cocaine injection decreases pGluA1-ser831 in the DM striatum regardless of cocaine experience. (A) pGluA1-ser831 protein expression was decreased in both cocaine-experienced rats and yoked-saline controls in the DM striatum after a cocaine challenge injection. There is no change in native GluA1 (B), pCaMKII-286 (C), or native CaMKII (D) protein expression in the DM striatum, regardless of cocaine experience. N = 4-7/group, "SA" = self-administration (The # represents a significant effect of challenge injection, $p<0.05$. There was no significant effect of pretreatment and no significant interaction.)

CHAPTER THREE

A CRITICAL ROLE FOR THE GLUA1 BINDING PARTNER, SAP97, IN COCAINE SEEKING

Samantha L. White[1], Pavel Ortinski[1], Shayna H Friedman[1], Rachael L. Neve[2], Robert G. Kalb[3], Heath D. Schmidt[1], R. Christopher Pierce[1*]

[1]Behavioral, Biochemical, Electrophysiological Experiments, Data Analysis, Manuscript:
Center for Neurobiology and Behavior
Department of Psychiatry
Perelman School of Medicine at the University of Pennsylvania
125 South 31st Street
Philadelphia, PA 19104

[2]HSV Vector Packaging: Department of Brain and Cognitive Sciences
McGovern Institute for Brain Research at the Massachusetts Institute of Technology
Building 46-3160, MIT
Cambridge, MA 02139

[3]miSAP97 Sequence Identification: Department of Neurology
Perelman School of Medicine at the University of Pennsylvania
Abramson Research Center 814
3625 Civic Center Blvd
Philadelphia, PA 19104

Abstract

A growing body of evidence indicates that the transport of calcium-permeable AMPA receptors (CP-AMPARs) to synapses in subregions of the nucleus accumbens promotes the reinstatement of cocaine seeking. Consistent with these findings, the present results show that administration of the CP-AMPAR blocker, Naspm, into the accumbens core or shell attenuated cocaine priming-induced reinstatement of cocaine seeking. Moreover, viral-mediated over-expression of "pore-dead" GluA1-containing AMPA receptors (via HSV GluA1-Q582E) into the core or shell attenuated the reinstatement of cocaine seeking, whereas over-expression of wild-type GluA1 subunits (via HSV GluA1-WT) in the shell, but not the core, enhanced the reinstatement of cocaine seeking. These results indicate that activation of GluA1-containing CP-AMPARs in accumbens subregions reinstates cocaine seeking. We next examined potential roles of SAP97 and 4.1N, proteins involved in GluA1 trafficking and stabilization at synapses, in this process. Viral-mediated expression of a microRNA that reduces SAP97 protein expression (HSV miSAP97) in the accumbens shell attenuated cocaine seeking. Viral expression and function were characterized by immunofluorescence and whole-cell patch clamp. In all cases, virus expression had no influence on the reinstatement of food seeking. Moreover, SAP97-mediated trafficking of GluA1-containing AMPA receptors to synapses in the accumbens shell is necessary for cocaine reinstatement. These results support investigation into GluA1 subunit accessory proteins as potential novel targets for pharmacotherapeutic intervention in cocaine craving and addiction.

Introduction

Although cocaine does not directly influence glutamate systems, repeated exposure to cocaine results in adaptations in glutamate transmission in limbic regions such as the nucleus accumbens. For example, during the reinstatement of cocaine seeking, an animal model of relapse, extracellular glutamate levels increase in the nucleus accumbens (Baker et al., 2003; McFarland et al., 2003). Moreover, administration of AMPA glutamate receptor antagonists into the nucleus accumbens attenuates cocaine priming-induced reinstatement, whereas activation of accumbens AMPA receptors promotes the reinstatement of cocaine seeking (Cornish et al., 1999; Cornish and Kalivas, 2000; Suto et al., 2004; Kruzich and Xi, 2006; Ping et al., 2008). The nucleus accumbens is divided into two major subregions, the core and shell. Increased AMPA receptors (AMPAR) transmission in both accumbal subregions of the nucleus accumbens contribute to the reinstatement of drug seeking precipitated by a cocaine priming injection (Schmidt and Pierce, 2010). Thus, microinjection of the AMPA receptor antagonist, CNQX, into either the core or shell attenuated cocaine priming-induced reinstatement (Famous et al., 2008) and administration of AMPA itself into either accumbens subregion elicited cocaine seeking (Ping et al., 2008).

There are four AMPA receptor subunits, GluA1-4, all of which become permeable to sodium and potassium upon activation (Pierce and Wolf, 2013). However, absence of GluA2 subunits from the AMPAR tetramer results in calcium permeability. GluA2-lacking calcium-permeable AMPARs (CP-AMPARs) have larger single channel conductance and faster kinetics relative to calcium-impermeable (CI) AMPARs that include GluA2 subunits (Tanaka et al., 2000; Cull-Candy et al., 2006; Lee, 2012). A growing body of evidence indicates that the balance of AMPARs in the nucleus accumbens is shifted to CP-AMPARs following repeated exposure to cocaine. Increased cell surface GluA1, but

not GluA2, expression was observed in the nucleus accumbens following cocaine self-administration (Anderson et al., 2008; Conrad et al., 2008). Electrophysiological studies demonstrated inward rectification of evoked AMPA receptor EPSCs, a hallmark of CP-AMPARs, in cocaine-experienced rats (Conrad et al., 2008; McCutcheon et al., 2011). These biochemical and electrophysiological results indicated an increase in CP-AMPARs (GluA1 homomers or GluA1/A3 receptors) in accumbens synapses after prolonged withdrawal. Supporting the functional significance of these findings, suppression of GluA1 transcription in either the accumbens core or shell impaired the reinstatement of drug seeking induced by a cocaine priming injection (Ping et al., 2008) and microinjection of the CP-AMPAR antagonist Naspm into the accumbens core blocked the expression of incubated cue-induced cocaine seeking (Conrad et al., 2008). Cocaine reinstatement also was attenuated by viral-mediated impairment of the transport of GluA1-containing AMPA receptors to synapses in the accumbens shell (Anderson et al., 2008). Together, these data indicate that increased glutamate transmission through accumbens shell CP-AMPARs promotes the reinstatement of cocaine seeking. Collectively, these findings suggest that synaptic incorporation of CP-AMPARs in the nucleus accumbens translates into enhanced drug seeking.

In order to expand upon these findings, we assessed the influence of Naspm administered into the core or shell on the reinstatement of cocaine seeking. We also used herpes simplex viral vectors to enhance or diminish the GluA1-mediated transmission in the core and shell prior to cocaine reinstatement. The mechanisms underlying the enhanced role of GluA1-containing AMPARs following cocaine exposure remain unclear. Multiple scaffolding proteins are involved in the trafficking and stabilization of GluA1 AMPAR subunits at the cell surface. For example, SAP97 is a PDZ domain-containing protein that assists in the trafficking of GluA1 subunits from

intracellular pools and the endoplasmic reticulum as well as the stabilization of GluA1-containing AMPARs the plasma membrane (Cai et al., 2002; Passafaro et al., 2001; Nikandrova et al., 2010) and 4.1N is an actin/spectrin binding protein that contributes to the trafficking and stabilization of GluA1-containing AMPARS at the cell surface (Lin et al., 2009). Interestingly, 4.1N also interacts with SAP97 to aid its targeting to the plasma membrane (Hanada et al., 2003). Using viral vectors strategies, we assessed the roles of accumbens SAP97 and 4.1N in the reinstatement of cocaine seeking.

Materials and Methods

Animals and Housing

Male Sprague-Dawley rats (*Rattus novergicus*) weighing 250-300 g were obtained from Taconic Laboratories (Germantown, NY, USA). Animals were individually housed with food and water available *ad libitum*. A 12h light/dark cycle was used and all experiments were performed during the light cycle. All experiments used Med-Associates (East Fairfield, VT, USA) operant chambers equipped with response levers, house light, and pumps for injecting drugs intravenously. Operant chambers were enclosed within ventilated, sound attenuating chambers. All experimental procedures were consistent with the ethical guidelines of the U.S. National Institutes of Health and were approved by the University of Pennsylvania School of Medicine Institutional Animal Care and Use Committee.

Drugs and Viruses

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Rockville, MD, USA) and dissolved in bacteriostatic 0.9% saline. We obtained 1-Naphthyl acetyl spermine trihydrochloride (Naspm) from Sigma-Aldrich (St. Louis, MO) and dissolved it in phosphate buffered saline (PBS). Dr. Rachael Neve at the MIT Viral Core packaged the viruses, GluA1-Q582E (Bachtel et al, 2008), GluA1-WT (Carlezon et al 1997), and 4.1N-CTD (Lin et al, 2009) into a Herpes Simplex Virus (HSV) p1005 plasmid, driven by an IE 4/5 promoter. The laboratory of Dr. Robert Kalb created the construct for miSAP97 (CAGTGACTGCCTTAAAGAATA) and scramble, and Dr. Rachel Neve at the MIT Viral Core also packaged these constructs into HSV. All viruses co-express green fluorescent protein (eGFP) driven under a separate CMV promoter.

Surgery

Rats were handled daily and allowed 1 week to acclimate to their home cages upon arrival. Prior to all surgeries, rats were anesthetized with 80 mg/kg ketamine (i.p.; Sigma-Aldrich, St. Louis, MO, USA) and 12 mg/kg xylazine (i.p.; Sigma-Aldrich, St. Louis, MO, USA). For cocaine self-administering animals, an indwelling silastic catheter (CamCaths, UK) was placed into the right jugular vein and sutured in place. The catheter was routed to a mesh backmount platform and implanted subcutaneously dorsal to the shoulder blades. Catheters were flushed daily with 0.3 ml of an antibiotic (Timentin, 0.93 mg/ml, Henry Schein, Melville, NY, USA) dissolved in heparinized saline. The catheters were sealed with plastic obturators when not in use. Following catheter insertion, we mounted animals in a stereotaxic apparatus and bilaterally implanted stainless steel guide cannulae (14 mm, 24 gauge) 2 mm dorsal to the medial nucleus accumbens shell, core or dorsal striatum. Dental acrylic affixed to stainless steel screws secured in the skull cemented the cannulae in place. The coordinates for the ventral ends of the guide cannulae, relative to bregma according to the atlas of Paxinos and Watson (1997) were as follows: shell = +1.0 mm A/P, ±1.0 mm M/L, –5.0 mm D/V; core = +1.0 mm A/P, ±2.5 mm M/L, –5.0 mm D/V; dorsal striatum = +1.0 mm A/P, ± 4.0 mm M/L, -5.0 mm D/V. In order to prevent occlusion, we inserted an obturator (14 mm, 33 gauge wire) into each guide cannula following surgery.

Animals used for food self-administration experiments had guide cannulae implanted into the nucleus accumbens core and shell, as described above. They did not receive a catheter.

Virus Injections

We mounted drug-naïve animals in a stereotaxic apparatus and 22 gauge Hamilton syringes holding 2.0 µl virus (according to experiment) were affixed to the stereotax and lowered bilaterally 0.5 mm dorsal to the medial nucleus accumbens shell and core. The coordinates for the ventral ends of the Hamilton syringes, relative to bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 1997) were as follows: shell = +1.0 mm A/P, ±1.0 mm M/L, –6.5 mm D/V; core = +1.0 mm A/P, ±2.5 mm M/L, –6.5 mm D/V. Virus was injected manually over the course of 10 minutes (0.2µl /minute) and the syringes were left in place for an additional 2 minutes to allow the virus to disperse. Upon recovering from anesthesia, animals were returned to their home cages until sacrificed for viral expression, biotinylation, or electrophysiological experiments.

Self-Administration and Extinction

Cocaine

Following a 7-day recovery period from surgery, rats were placed in operant chambers and allowed to lever press for intravenous cocaine infusions (0.25 mg/59 µl saline over 5 s) over a 2-hour time period daily for 21 days. Each self-administration session began with the i.v. administration of 59 µl cocaine (0.25 mg) to fill the catheter (little or none of this non-contingent injection reached the systemic circulation). Rats were initially trained using a fixed ratio (FR) 1 schedule of reinforcement (limited to 30 injections per 120 min). Once animals achieved stable responding, as defined by at least 20 infusions over the 2 h session for 2 consecutive days, they were transitioned to an FR5 schedule of reinforcement (limited to 30 injections per 120 min). For both schedules of reinforcement, a 20s time-out period followed each cocaine infusion, during which time

active, drug-paired lever responses were tabulated but had no scheduled consequence. Each operant chamber was also equipped with an inactive lever. Responses made on the inactive lever, which had no scheduled consequence, were also recorded during all training sessions.

Following cocaine self-administration, drug-taking behavior was extinguished by replacing cocaine with 0.9% bacteriostatic saline. Extinction continued until responding on the drug-paired lever was <15% of the response rate on the last day of cocaine self-administration using the FR5 schedule of reinforcement.

Sucrose

After a 7-day recovery period, we food deprived (4 pellets/day, 80% body weight) the rats and trained them to administer sucrose pellets on a fixed ratio (FR) 1 schedule, where they received one food pellet each time they pressed the active lever (Bio-Serv, Frenchtown, NJ). After receiving over 20 pellets per session over two consecutive self-administration sessions, the rats then advanced onto a FR 5 schedule where they received one sucrose pellet after pressing the active lever five times. Rats self-administered sucrose pellets for a total of 14 days with a maximum of 30 pellets per session.

Following sucrose self-administration, sucrose-seeking behavior was extinguished where active lever pressing resulted in no sucrose pellets. Extinction of sucrose-seeking behavior continued on an FR5 schedule until responding on the active lever was 15% or less of that on the last self-administration day.

Microinjections for Reinstatement Experiments

Naspm

We removed the obturators from the guide cannulae and inserted 33 gauge stainless steel microinjectors (Small Parts Inc) into the guide cannula. We cut microinjectors to a length that extended 2 mm below the ventral end of the guide cannulae, and lowered the microinjectors into the medial nucleus accumbens shell, accumbens core, or dorsal striatum. Bilateral infusions occurred over a 120 s time period for a total volume of 0.5 µl of Naspm (40µg) or saline per hemisphere. This dose has been previously confirmed in the literature to produce behavioral effects (Conrad et al., 2008). Following microinfusion of either Naspm or saline, the microinjectors remained in place for 60 s in order to allow the solution to diffuse away from the tips of the microinjectors before removal. 10 min following microinjections we administered a systemic priming injection of cocaine (10 mg/kg, i.p.) or experimenter-delivered sucrose pellets and began the reinstatement session (see below).

The goal of the experimental design was to have each animal serve as its own control and receive up to two microinjections per brain region (i.e. one dose of Naspm plus saline for a maximum of two microinjections per brain region). However, we were frequently forced to deviate from this experimental design when technical difficulties (i.e. blocked microinjection cannula or loss of catheter patency) made it impossible of Naspm plus vehicle in each brain region. Therefore, we used a mixed-factors design in these behavioral experiments. In every case, however, an animal received at a minimum, treatment of one drug dose and its vehicle. In order to control for potential order effects of drug and vehicle administrations, all drug and vehicle treatments were counterbalanced across reinstatement sessions.

Using this experimental design, subjects underwent a series of alternating extinction and reinstatement sessions that lasted for approximately 16 days. During this period, extinction of the ability of priming injections of cocaine to induce reinstatement is a concern. However, we have previously shown that reinstatement of cocaine seeking persists for at least 20 days after the initial extinction of cocaine self-administration behavior (Anderson et al., 2008; Famous et al., 2008).

Viral Vector

Only one viral vector was microinjected bilaterally per animal in a between-subjects design. We removed the obturators from the guide cannulae and inserted 33 gauge stainless steel microinjectors (Small Parts Inc). We cut microinjectors to a length that extended 2 mm below the ventral end of the guide cannulae and lowered them into the medial nucleus accumbens shell or core. Bilateral infusions of HSV GluA1-Q582E, GluA1-WT, 4.1N-CTD, eGFP, miSAP97, or scramble occurred simultaneously over a 10 min time period for a total volume of 2.0 µl per side. Following the injections, we left microinjectors in place for 120 s to allow the solution to diffuse away from the tips of the cannula before they were removed. Animals underwent daily extinction sessions until reaching the first reinstatement test session 3 days (GluA1-Q582E, GluA1-WT, 4.1N-CTD, eGFP) or 4 days (miSAP97, scramble) post-infusion (see below). Animals in viral vector experiments were then maintained on extinction sessions until the second reinstatement test at 1-week post-injection.

Cocaine Priming-induced Reinstatement

Upon reaching extinction criteria, animals received microinjections of Naspm or viral vectors as previously described (see Table 3A for cohort details). Immediately prior to the reinstatement test session, animals received a systemic priming injection of 10

mg/kg cocaine or 5 mg/kg cocaine (i.p.). Following the priming injection of cocaine, we placed animals in the operant chamber for a 2 h reinstatement session under extinction conditions (i.e. active lever presses resulted in an infusion of saline instead of cocaine). We recorded active and inactive lever responding. Extinction sessions followed each reinstatement session until responding was again less than 15% of the response rate maintained by cocaine self-administration.

Sucrose Reinstatement

Upon reaching extinction criteria, animals received microinjections of Naspm or viral vectors as previously described (see Table 3B for cohort details). To assess sucrose reinstatement in all groups, we placed the animals in the operant chambers and the session began with a prime of two non-contingent sucrose pellets. Over the first 10 min of the reinstatement session, the experimenter remotely administered two non-contingent sucrose pellets every 2 minutes. Active lever responding was recorded for the entire 60-minute session. Extinction sessions followed each reinstatement session until responding was again less than 15% of the response rate maintained by sucrose self-administration.

Verification of Cannulae Placements

After the completion of all behavioral experiments, the animals were given an overdose of pentobarbital (100 mg/kg) and then the brain was removed and stored in formalin for at least three days. Subsequently, coronal sections (100 μm) were taken at the level of the nucleus accumbens with a Vibratome (Technical Products International; St. Louis, MO). The sections were mounted on gelatin-coated slides. An individual unaware of the animals' behavioral response determined cannula placements as well as potential drug- or cannula-induced neuronal damage. Cellular death and associated gliosis also were

assessed. Animals with cannulae placements outside of the areas of interest, or with excessive mechanical damage, were excluded from subsequent data analysis.

Viral Expression

To ascertain viral expression via green fluorescent protein, we injected (2μl/side) HSV GluA1-Q582E, HSV 4.1N-CTD, HSV GluA1-WT, or HSV miSAP97 into the nucleus accumbens of separate, drug-naïve rats (N=10). At 3 days, 4 days, or one week after injection, animals received 100 mg/kg pentobarbital (i.p.) before perfusion with 120mL ice-cold PBS followed by 60 mL 4% PFA dissolved in ice-cold PBS. Brains were removed and placed in 4% PFA for 24 hours before storage in 30% sucrose dissolved in PBS with 1% sodium azide. Experimenters sliced 10-30μm coronal sections on a cryostat (Cryotome FE, Thermo) and mounted them directly onto polarized glass slides. Dry slides were washed in 1X PBS, and then blocked for one hour in 0.1% triton and 3% normal donkey serum in 1X PBS. We then added primary antibody (Anti-eGFP, Ms/Rb Millipore, diluted 1:1000 in 0.1% triton + 3% donkey serum in PBS) to the slides and incubated overnight at 4°C. The next day, experimenters washed the slides in 1X PBS before incubating in secondary fluorescent antibody at room temperature for 2 hours. Secondary fluorescent antibodies included: Anti-Rabbit and Anti-Mouse Alexa Fluor 488 (1:500, Jackson Immuno Research). After 2 hours, slides were washed in 1X PBS before being cover-slipped using Vectashield mounting medium (Vector Laboratories) and imaged for eGFP expression using fluorescent microscopy.

Electrophysiology

Separate, drug-naïve rats received bilateral injections of HSV GluA1-Q582E, HSV GluA1-WT, or HSV miSAP97 (2μl) into the accumbens shell. After 3- or 4-days, animals were anesthetized with isofluorane in preparation for electrophysiological experiments as described previously (Ortinski et al., 2012). Briefly, the brain was removed and coronal

slices (300 mm) containing the nucleus accumbens were cut with a Vibratome (VT1000S, Leica Microsystems) in ice-cold artificial cerebrospinal fluid solution (ACSF), in which NaCl was replaced by an equiosmolar concentration of sucrose. ACSF consisted of 130mM NaCl, 3mM KCl, 1.25mM NaH2PO4, 26mM NaHCO3, 10mM glucose, 1mM MgCl2, and 2mM CaCl2 (pH 7.2–7.4 when saturated with 95% O2/5% CO2). Slices were incubated in ACSF at 32–34 1C for 45 min and kept at 22–25 1C thereafter, until transfer to the recording chamber. Slices were viewed using infrared differential interference contrast optics under an upright microscope (Eclipse FN1, Nikon Instruments) with a 40X water immersion objective.

For evoked EPSCs (eEPSCs), a minimal stimulation intensity required to evoke a consistent post-synaptic response was determined. Peak eEPSC amplitudes were then measured at double and triple the minimal stimulation intensity (at 0.05-0.1 Hz) to construct the input/output diagrams. Average responses were then calculated based on 5-10 eEPSCs at each stimulation intensity. The rectification index of AMPA receptor-mediated eEPSCs was measured as the ratio of the peak current amplitude at +40 mV to the peak current amplitude at -70 mV. For rectification index, the measurements were based on 5-10 eEPSCs evoked at 0.1 Hz.

Western Blot

For HSV 4.1N-CTD protein expression, drug-naïve animals received unilateral injections of HSV 4.1N-CTD or HSV eGFP (2μl) into each hemisphere of the accumbens shell (N=10/virus) and were returned to recover in their home cage for 3 days, respectively. To measure knock down of SAP97 protein expression due to HSV miSAP97, drug-naïve animals received bilateral injections of HSV miSAP97 or HSV Scramble into the core and shell of the nucleus accumbens (N=6/virus) and were returned to recover in their

home cage for 4 days. Following rapid, live decapitation, brains were extracted and flash-frozen in isopentane (-80 C). Experimenters used a cryostat to dissect out the nucleus accumbens before processing whole-cell tissue for Western blot as described previously (Anderson *et al*., 2008).

For all samples, protein concentration was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (10-20 μg) were loaded and separated in 10% Tris-Glycine gels (Invitrogen) under reducing conditions, then transferred to nitrocellulose membranes using the i-Blot dry transfer system (Invitrogen). Membranes were blocked with Odyssey Blocking Buffer (Invitrogen), according to manufacturer instructions. Membranes were incubated overnight at 4° C with selective antibodies to: SAP97 (Rb 1:1000, AbCam), 4.1N (Rb 1:1000, Millipore), and GAPDH (1:2000, Cell Signaling) diluted in Odyssey Blocking Buffer. After washing with TBST, membranes were then incubated with infrared secondary antibodies at room temperature for 1 hr (1:5000, IR-dye 680 or IR-dye 800), before being imaged on an Odyssey fluorescent scanner (Licor Biosciences). To ensure equal loading, GAPDH expression was used as a loading control and all proteins were normalized to GAPDH integrated intensity values.

Results

Blocking CP-AMPARs in the accumbens core or shell, but not dorsolateral striatum, selectively attenuates cocaine priming-induced reinstatement of drug seeking

In order to assess the role of CP-AMPARs in the reinstatement of cocaine seeking, a CP-AMPAR antagonist (Naspm) was microinjected into regions of the striatal complex that contribute to various aspects of cocaine-seeking behavior. Total active and inactive lever responding (mean ± SEM) during the reinstatement of cocaine seeking following microinjection of saline or 40 µg Naspm into the accumbens core, accumbens shell or DL striatum are shown Figures 3.1A, 3.1C and 3.1E. Reinstatement data were analyzed with three separate analyses of variance (ANOVAs) with both drug treatment and lever as the between subjects factors in all cases. Analysis of the core data revealed significant main effects of treatment ($F_{1,11}$=11.98, p<0.0053) and lever ($F_{1,11}$=57.02, p<0.0001) as well as a significant treatment × lever interaction ($F_{1,11}$=11.72, p<0.0057). *Post hoc* tests revealed a significant difference in active lever responding between treatments (Bonferroni, p<0.05, Figure 3.1A). Analysis of the shell data showed significant main effects of treatment ($F_{1,7}$=16.51, p<0.0048) and lever ($F_{1,7}$=47.76, p<0.0002) as well as a significant drug × lever interaction ($F_{1,7}$=22.37, p<0.0021). Pairwise comparisons demonstrated a significant difference in active lever responding between treatments (Bonferroni, p<0.05, Figure 3.1C). Analysis of the DL striatum data, which is shown in Figure 3.1E, showed a significant main effect of lever ($F_{1,3}$=22.0, p<0.0186) but no significant main effect of treatment ($F_{1,3}$=0.004, p<0.9538) and no significant treatment × lever interaction ($F_{1,3}$=0.0063, p<0.9417).

Decreases in responding, such as following intra-core or shell Naspm, could be due to generalized behavioral disruption. Although Naspm produced no effect on

inactive lever responding, the low average number of inactive responses per session decreases the utility of this measure as a means to judge drug-induced behavioral suppression. Therefore, we also assessed the reinstatement of sucrose seeking. Total responses on the active lever (mean ± SEM) during the sucrose reinstatement sessions are summarized in Table 3.1. Microinjection of Naspm into the shell had no influence on the reinstatement of sucrose seeking (p<0.8221). However, intra-core Naspm significantly attenuated sucrose seeking (p<0.0104), which indicates that CP-AMPARs in the core may have a more generalized influence on behaviors motivated by cocaine as well as natural reinforcers.

Over-expression of "pore dead" GluA1 subunits in the core and shell reversibly attenuates cocaine seeking

We initially characterized HSV GluA1-Q582E (pore dead GluA1) electrophysiologically in naïve rats (i.e. not exposed to cocaine) three days after microinjection of the virus into the nucleus accumbens. Representative examples of eEPSCs recorded at minimum stimulation intensity (1X) as well as double (2X) and triple (3X) minimum stimulation intensity are shown in Figure 3.2A (left). Quantification of this experiment is depicted in Figure 3.2B. These data were analyzed with a two-way ANOVA, which revealed significant main effects of treatment ($F_{1,9}$=11.69, p<0.0076) and stimulation intensity ($F_{2,18}$=15.33, p<0.0001) as well as a significant interaction between these factors ($F_{2,18}$=10.96, p<0.0008). Subsequent pairwise tests showed a significant difference between amplitudes measured from eGFP negative and eGFP positive cells at the 3X stimulation intensity (Bonferroni, p<0.05, Figure 3.2B). The Rectification Index (RI) was measured as the ratio of the peak current amplitude at +40 mV to the peak current amplitude at -70 mV in accumbens eGFP negative and eGFP positive neurons, as

illustrated in Figure 3.2A (right). Quantification of this experiment is depicted in Figure 3.2C. These data were analyzed with a *t*-test (unpaired, two-tailed), which revealed a significant effect of GluA1-Q582E expression on RI (t_6=3.068, p<0.022).

Next, we examined the effect of HSV GluA1-Q582E administered into the core or the shell on the reinstatement of cocaine seeking. As summarized in the experimental design outlined in Figure 3.3A the first reinstatement session occurred three days after the virus microinjection (HSV eGFP or HSV GluA1-Q582E), which is when HSV expression peaks (see Figure 3.3B). Total active and inactive lever responding during the first reinstatement session for animals administered viruses into the core or shell are depicted in Figures 3.3C and 3.3D, respectively (the DL striatum was not examined due to the negative Naspm results summarized previously). Reinstatement was prompted by administration of 10 mg/kg cocaine (i.p.). These data were analyzed with separate mixed factors ANOVAs with virus as the between subjects factor and lever and the within subjects factor. Analysis of the core data showed significant main effects of virus ($F_{1,15}$=9.629, p<0.0073) and lever ($F_{1,15}$=42.11, p<0.0001) as well as a significant treatment × lever interaction ($F_{1,15}$=10.72, p=0.0051). Analysis of the shell data revealed significant main effects of virus ($F_{1,19}$=30.02, p<0.0019) and lever ($F_{1,19}$=30.51, p<0.0001) as well as a significant treatment × lever interaction ($F_{1,19}$=10.75, p<0.0039). Subsequent pairwise analyses revealed significant differences between active lever virus effects for both the core and the shell (Bonferroni, p<0.05). Sucrose reinstatement also was assessed 3 days after virus administration in a separate cohort of animals. The data, which are summarized in Table 3.1, indicated no significant differences between HSV eGFP and HSV GluA1-Q582E when administered into either the core (p<0.6299) or shell (p<0.8180).

As shown in Figure 3.3E, HSV is dormant 7 days after administration. A second reinstatement session was performed at this time point and the results were analyzed the same as described above for reinstatement session 1. Analyses indicated only significant main effects of lever for both the core ($F_{1,9}$=143.5, p<0.0001, Figure 3.3F) and shell ($F_{1,11}$=28.23, p<0.0002, Figure 3.3G). Taken together, these results show that the peak expression of pore-dead GluA1 subunits in either the core or the shell attenuated cocaine seeking. Equivalent levels of cocaine reinstatement were observed during the second reinstatement session, at which point the virus was dormant.

Over-expression of GluA1 subunits in shell, but not the core, promotes cocaine seeking

In these experiments HSV eGFP or HSV GluA1-WT were microinjected into the core or shell using the same paradigm as depicted in Figure 3.3 with the exception that a subthreshold dose of cocaine (5 mg/kg, i.p.) was used in the reinstatement sessions. The statistical analyses were the same as used in Figure 3.3. The analysis of the core data indicated no significant main effects or interactions at either reinstatement session (see Figures 3.4A and 3.4C). For the shell, analysis of the first reinstatement session indicated a significant main effect of treatment ($F_{1,15}$=13.97 p<0.002), a significant main effect of lever ($F_{1,15}$=46.25 p<0.0001) and a significant treatment × lever interaction ($F_{1,15}$=8.452 p<0.0108). *Post hoc* tests revealed a significant active lever difference between treatments (Bonferroni, p<0.05, Figure 3.4B). For the second reinstatement test, there was a significant main effect of lever ($F_{1,14}$=8.446 p<0.0115) but no other significant main effect or interaction (see Figure 3.4D). These data indicate that a normally subthreshold dose of cocaine induced cocaine seeking when GluA1 subunits were over-expressed in the accumbens shell, but not the core.

Inhibiting the interaction between 4.1N and GluA1 in the shell has no effect on cocaine seeking

The next experiments examined potential mechanisms underlying the influence of prior cocaine exposure on GluA1-containing AMPAR-mediated transmission. Since the most consistent results of the previously described experiments were obtained with shell manipulations, we focused our attention of this accumbal subregion. We first tested the virus in naïve rats and confirmed that eGFP expression in the nucleus accumbens peaked 3 days following administration (see Figure 3.5A) and was absent seven days after microinjection (data not shown). 4.1N protein expression was significantly increased 3 days after HSV 4.1N-CTD (t_{18}=2.694, p<0.0148, Figure 3.5B).

Cocaine priming-induced reinstatement was assessed at two time points, as described previously. Analysis of the reinstatement data revealed only a significant main effects of lever responding for the first ($F_{1,19}$=47.30 p<0.0001, Figure 3.5C) and second ($F_{1,10}$=20.37 p=0.0011, Figure 3.5D) reinstatement sessions. Thus, over-expression of 4.1N-CTD in the accumbens shell had no influence on the reinstatement of cocaine seeking.

Reduced expression of SAP97 in the accumbens shell attenuates cocaine priming-induced reinstatement

HSV miSAP97 was initially tested in naïve rats. Knockdown of SAP97 protein by over-expression of miSAP97 *in vivo* (Figure 3.6A) was assessed using western blot. SAP97 protein expression levels were analyzed using a *t*-test (unpaired, two-way). Analysis of *in vivo* injection of miSAP97 in the accumbens shell and core revealed a trend towards a 30% decrease of SAP97 protein expression compared to HSV Scramble injection (t(9)=1.719 p=0.1197, Figure 3.6A). We have initiated electrophysiological

characterization of HSV miSAP97. Preliminary electrophysiological analysis of peak eEPSC amplitude (mean ± SEM) recorded at minimum stimulation intensity (1X) as well as double (2X) and triple (3X) minimum stimulation intensity is shown in Figures 3.6B. eEPSC amplitudes were analyzed using a two-way ANOVA. In miSAP97 expressing cells, preliminary analysis of eEPSC amplitude across 3 stimulation intensities revealed no effect of treatment (p=0.2574), a significant main effect of stimulation intensity ($F_{2,22}$=14.46, p<0.0001), and no treatment × intensity interaction ($F_{2,22}$=1.71, p=0.2039, Figure 3.6B).

As summarized in the experimental design, the first reinstatement session occurred four days after the virus microinjection (HSV eGFP or HSV miSAP97), to allow sufficient time for SAP97 knock down by miSAP97 (see Figure 3.7A). With this virus, the peak effect was observed 4 days after administration of HSV miSAP97 into the nucleus accumbens shell (Figure 3.7B). The virus was dormant 7 days after microinjection (see Figure 3.7C). The 4 and 8-day time points were used for the two reinstatement sessions. Analysis of the reinstatement data collected during the first session revealed significant main effects of treatment ($F_{1,16}$=8.225, p<0.0112) and lever ($F_{1,16}$=57.19, p<0.0001) and a significant treatment × lever interaction ($F_{1,16}$=7.107, p<0.01). *Post hoc* tests revealed a significant active lever difference between treatments (Bonferroni, p<0.05, Figure 3.7D). Analysis of the second reinstatement session showed only a significant main effect of lever ($F_{1,11}$=90.56, p<0.0001) with no other significant main effect or interaction (see Figure 3.7E). The reinstatement of sucrose seeking also was assessed. HSV miSAP97 had no effect on sucrose seeking (p<0.4717, see Table 3.1).

Discussion

The present results revealed that administration of the CP-AMPAR blocker, Naspm, into the shell of the nucleus accumbens attenuated the reinstatement of cocaine seeking. In the nucleus accumbens, CP-AMPARs are predominantly GluA1 homomers or GluA1/GluA3 heteromers (Pierce and Wolf, 2013). The current findings showed that viral-mediated impairment of GluA1 function in the accumbens shell attenuated cocaine reinstatement, whereas viral-mediated over-expression of GluA1 subunits in the shell enhanced cocaine seeking. In order to examine mechanisms underlying the enhanced influence of CP-AMPARs during the reinstatement of cocaine seeking, a viral vector was used to impair the interaction between AMPARs and SAP97, a protein involved in the trafficking of GluA1-containing AMPARs to the cell surface. Reduced shell expression of SAP97 attenuated the reinstatement of drug-seeking behavior. Collectively, these results indicate that SAP97 plays a critical role in functioning of CP-AMPARs in the accumbens shell during the reinstatement of cocaine seeking.

CP-AMPARs and Cocaine Reinstatement

It was previously shown that administration of Naspm into the accumbens core blocked enhanced ("incubated") cue-induced reinstatement following extended access cocaine self-administration and protracted forced abstinence (Conrad et al., 2008). Consistent with this finding, we showed that Naspm administered into either the accumbens core or the shell (but not the DL striatum) attenuated the reinstatement of cocaine seeking induced by a cocaine priming injection. Administration of Naspm into the core also impaired sucrose seeking, suggesting that CP-AMPARs in the core may serve a broader role in the modulation of reinforced behaviors. The shell effect was reinforcer-specific in that intra-shell Naspm had no influence on sucrose reinstatement. Taken together,

these results indicate that CP-AMPARs in the shell play a specific role in cocaine priming-induced reinstatement of drug seeking following limited access (2-hour/day) cocaine self-administration.

GluA1-containing AMPARs and Cocaine Reinstatement

In order to examine the influence of GluA1-containing AMPARs specifically, pore dead GluA1-Q582E was over-expressed in the core or shell. Impairing GluA1 AMPAR function in both the core and shell attenuated cocaine priming-induced reinstatement, without altering sucrose seeking behaviors. Correspondingly, the present results showed that viral-mediated over-expression of wild type GluA1 in the shell promoted drug seeking when combined with a subthreshold dose of cocaine. HSV-GluA1-Q582E administered into the accumbens core was previously shown to enhance the reinstatement of cocaine seeking induced by a cocaine priming injection or intra-core AMPA, while core over-expression of WT GluA1 produced the opposite effect (Bachtell et al., 2008). These researchers used a paradigm that incorporated abstinence periods between the self-administration and extinction phases (Bachtell et al., 2008), which is not typical in priming-induced reinstatement experiments. Moreover, rats were food trained prior to cocaine self-administration, which makes it difficult to determine if the reinstatement was drug seeking alone or combined with food seeking. It is not clear if methodological differences such as this explain these results. Regardless, these findings oppose the consensus view that activation of accumbens GluA1-containing AMPA receptors promotes the reinstatement of cocaine seeking, which is consistent with the current results. Numerous studies have shown that AMPAR antagonists microinjected into the core or shell impairs cocaine seeking (Famous et al., 2008; Ping et al., 2008; Cornish et al., 2000; Park et al., 2002). Moreover, decreased GluA1-

containing AMPAR transmission in the core or shell disrupts the reinstatement of cocaine seeking (Anderson et al., 2008; Ping et al., 2008; Conrad et al., 2008).

GluA1 Accessory Proteins and AMPA Receptor Function

Synapse-associated protein 97 (SAP97) and protein 4.1N are two of many accessory proteins involved in the trafficking, stabilization, and function of GluA1-containing AMPA receptors at the cell surface (Lisman et al., 2012). The interaction between the C-terminal domain (CTD) of 4.1N and the membrane proximal region of GluA1 subunits aids their insertion into the cell membrane *in vitro* (Shen et al., 2000; Lin et al., 2009). Additionally, binding of the N-terminal Four.1-Ezrin-Radixin-Moesin (FERM) domain in 4.1N with the I3 insert of SAP97 aids the targeting of SAP97 to the cell surface (Lue et al., 1994; Hanada et al., 2003). A multitude of *in vitro* experiments identify a role for SAP97 interactions with GluA1 subunits, seen in the rate of subunit insertion into the cell surface, GluA1 spine clustering and dendritic growth, and AMPAR-mediated mini EPSCs (Passafaro et al., 2001; Mauceri et al., 2004; Zhou et al., 2008; Waites et al., 2009). These consequences of SAP97-GluA1 subunit interactions are mediated by post-translational modifications mediated by the serine/threonine kinase, CaMKII (Schluter et al., 2006). Interestingly, cocaine priming-induced reinstatement activates CaMKII (Anderson et al., 2008), which phosphorylates both SAP97 and GluA1 subunits to regulate their targeting and function (Mauceri et al., 2004).

Our results indicated that disrupting the interaction between 4.1N and GluA1-containing AMPARs had no influence on cocaine priming-induced reinstatement. When we virally over-expressed only the CTD of 4.1N in the accumbens shell, animals displayed robust reinstatement of drug-seeking behavior in response to systemic cocaine. Our original hypothesis predicted that the virus would reduce GluA1-containing

AMPAR surface expression because GluA1 subunits would be bound by the recombinant 4.1N-CTD that lacked the domains necessary to interface with the plasma membrane or actin/spectrin cytoskelton. Given our previous results, such a manipulation should attenuate the reinstatement of cocaine-seeking behaviors. However, expression of 4.1N-CTD had no effect on endogenous 4.1N or SAP97, leaving endogenous 4.1N available to interact with SAP97 that can freely bind GluA1, potentially protecting the trafficking and function of GluA1 subunits indirectly (Rumbaugh et al., 2003).

We found that transient knock down of SAP97 in the accumbens shell reduced AMPAR function in MSNs in a manner similar to pore dead GluA1-Q582E. While there is some evidence that SAP97 binding to the PDZ motif in the extreme C-terminus of GluA1 alters receptor trafficking, others find no change in GluA1 trafficking due to mutations of the C-tail or over-expression of SAP97 (Passafaro et al., 2001; Mauceri et al., 2004; Zhou et al., 2008). In agreement with literature indicating that SAP97 is necessary for GluA1 subunit trafficking, we measured a trend towards decreased AMPAR-mediated EPSCs and decreased GluA2-lacking AMPAR currents in infected cells. Our results demonstrated novel, drug-specific effects on reinstatement behaviors after expression of a microRNA against SAP97 in the accumbens shell. Knockdown of SAP97 in the accumbens shell, presumably due to disrupted functioning of CP-AMPARs, attenuated cocaine priming-induced reinstatement. Reduced SAP97 expression in the shell did not influence the reinstatement of food-seeking behavior, suggesting that the knockdown did not have a generalized effect on behavior or natural rewards. These findings are in agreement with our Naspm and GluA1-Q582E data in that all three experiments disrupted shell CP-AMPAR function, which impaired cocaine priming-induced reinstatement of drug seeking without affecting food seeking. Taken

together, these results indicate that SAP97 plays a necessary role in the up-regulation of CP-AMPARs in the accumbens shell following cocaine self-administration.

Conclusions

These experiments have shown that excitatory transmission through CP-AMPARs in the nucleus accumbens shell mediates the reinstatement of cocaine-seeking behavior. Knockdown of the GluA1 accessory protein SAP97 is sufficient to reduce AMPAR currents, particularly those via GluA2-lacking AMPARs, in accumbens MSNs. Additionally, knockdown of SAP97 in the accumbens shell attenuated cocaine priming-induced reinstatement of drug seeking. Taken together, these findings indicate that SAP97 interactions with GluA1 subunits in the shell are necessary to maintain functionally up-regulated CP-AMPARs that are required for the reinstatement of cocaine seeking. Further, these data identify SAP97 as a potential novel target for pharmacotherapeutic intervention in cocaine craving and addiction.

TABLE 3A

Treatment	Region	N	Cocaine (i.p.)	Time post-injection
Naspm/Saline	Shell	8	10 mg/kg	10 min
	Core	13	10 mg/kg	10 min
	Dorsal Striatum	5	10 mg/kg	10 min
GluA1-Q582E	Shell	6-8	10 mg/kg	3 days, 1 week
	Core	6-8	10 mg/kg	3 days, 1 week
GluA1-WT	Shell	9	5 mg/kg	3 days, 1 week
	Core	8-9	5 mg/kg	3 days, 1 week
4.1N	Shell	5-8	10 mg/kg	3 days, 1 week
eGFP	Shell	7-13	5 or 10 mg/kg	3 days, 1 week
	Core	7-9	5 or 10 mg/kg	3 days, 1 week
miSAP97	Shell	6-9	10 mg/kg	4 days, 1 week
Scramble	Shell	7-9	10 mg/kg	4 days, 1 week

Cocaine priming-induced reinstatement experimental cohorts. The microinjection treatment, brain region, animals used, cocaine dose (i.p.), and time of reinstatement test following microinjection are as indicated.

TABLE 3B

Treatment	Region	N	Time post-injection
Naspm/Saline	Shell	8	10 min
	Core	5	10 min
GluA1-Q582E	Shell	6	3 days, 1 week
	Core	6	3 days, 1 week
eGFP	Shell	6	3 days, 1 week
	Core	6	3 days, 1 week
miSAP97	Shell	6	4 days, 1 week
Scramble	Shell	6	4 days, 1 week

Non-contingent sucrose reinstatement experimental cohorts. The microinjection treatment, brain region, animals used, and time of reinstatement test following microinjection are as indicated.

TABLE 3.1

Treatment	Region Injected	Reinstatement 1 (Active Lever)	Statistics
Saline	Core	85.80±14.10	t_8=3.327, p<0.0104*
Naspm	Core	28.60±9.826	
Saline	Shell	77.5±13.19	t_{14}=0.2291, p<0.8221
Naspm	Shell	81.25±9.689	
Control	Core	77.67±16.64	t_{10}=0.4970, p<0.6299
Pore Dead GluA1	Core	89.83±17.96	
Control	Shell	117.0±24.46	t_9=0.2370, p<0.8180
Pore Dead GluA1	Shell	125.2±23.93	
Control	Shell	117.0±36.88	t_{10}=0.7485, p<0.4714
miSAP97	Shell	158.0±40.5	

The effects of intra-core or –shell pharmacological and viral vector-mediated manipulation of CP-AMPAR transmission on the reinstatement of sucrose seeking. Summary of findings across sucrose reinstatement experiments indicating microinjection, region, and total active lever responding (mean ± SEM). All data were analyzed using a two-tailed t Test. Intra-core microinjection of Naspm significantly attenuated sucrose-seeking behavior compared to saline controls. * indicates p<0.05

FIGURE 3.1

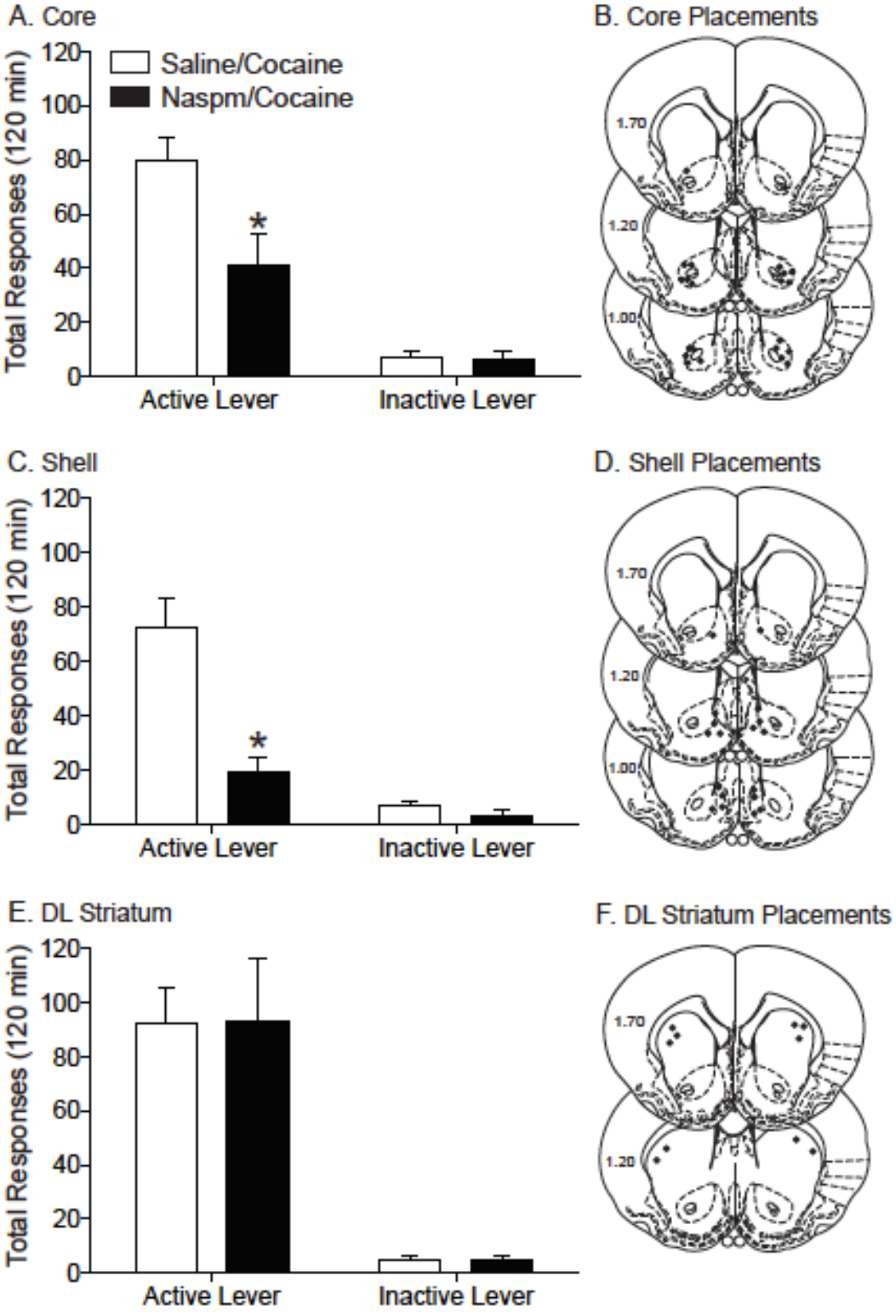

A. Core
Saline/Cocaine
Naspm/Cocaine
Total Responses (120 min)
120
100
80
60
40
20
0
*
Active Lever
Inactive Lever
B. Core Placements
1.70
1.20
1.00
C. Shell
Total Responses (120 min)
120
100
80
60
40
20
0
*
Active Lever
Inactive Lever
D. Shell Placements
1.70
1.20
1.00
E. DL Striatum
Total Responses (120 min)
120
100
80
60
40
20
0
Active Lever
Inactive Lever
F. DL Striatum Placements
1.70
1.20

Figure 3.1: Microinfusion of CP-AMPAR antagonist Naspm into the accumbens core or shell, but not the DL striatum, attenuates cocaine priming-induced reinstatement. The data are expressed as the mean (± SEM) lever responding on the active and inactive levers during the 2-hour reinstatement test sessions. A) Total lever responding following intra-core microinfusions of saline or Naspm (40µg/side, n=12/group) 10 minutes prior to a systemic injection of cocaine (10 mg/kg, i.p.). Bonferroni *post hoc p*<0.05 for active lever responding between saline and Naspm treatments. B) Cannula placements in the core of the nucleus accumbens. The numbers indicate mm anterior to bregma according to Paxinos and Watson (1997). C) Total lever responding following intra-shell microinfusions of saline or Naspm (40µg/side, n=8/group) 10 minutes prior to a systemic injection of cocaine (10 mg/kg, i.p.). Bonferroni *post hoc p*<0.05 for active lever responding between saline and Naspm treatments. D) Cannula placements in the shell of the nucleus accumbens. E) Total lever responding following intra-DL striatum microinfusions of saline or Naspm (40µg/side, n=5/group) 10 minutes prior to a systemic injection of cocaine (10 mg/kg, i.p.). F) Cannula placements in the DL striatum of the nucleus accumbens.

FIGURE 3.2

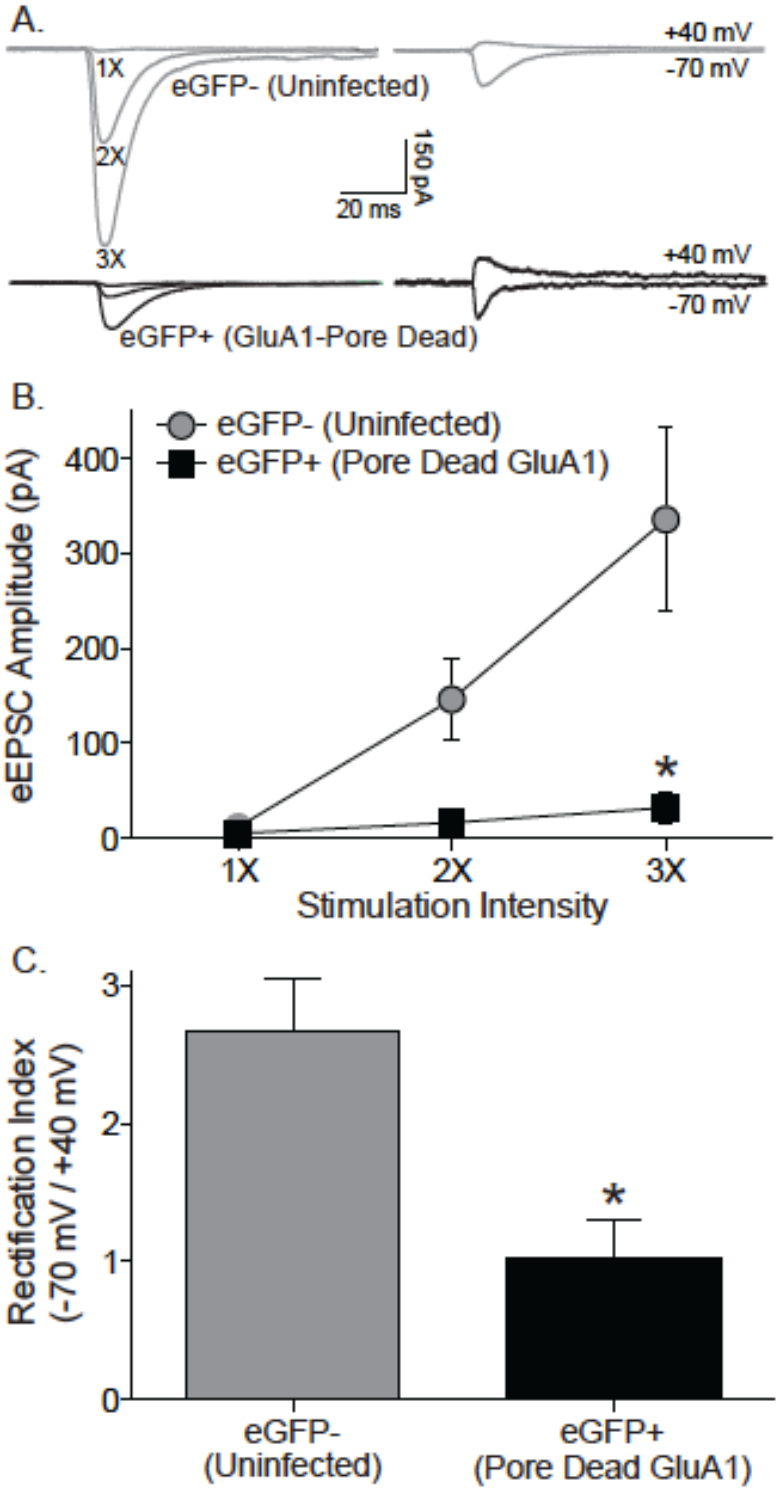

A.
1X
2X
3X
eGFP- (Uninfected)
+40 mV
-70 mV
150 pA
20 ms
+40 mV
-70 mV
eGFP+ (GluA1-Pore Dead)
B.
eGFP- (Uninfected)
eGFP+ (Pore Dead GluA1)
eEPSC Amplitude (pA)
400
300
200
100
0
*
1X
2X
3X
Stimulation Intensity
C.
Rectification Index
(-70 mV / +40 mV)
3
2
1
0
*
eGFP-
(Uninfected)
eGFP+
(Pore Dead GluA1)

Figure 3.2: Over-expression of GluA1-Q582E in medium spiny neurons attenuates synaptic strength and decreases inward rectification of AMPAR eEPSCs. Infected cells (eGFP+ Pore dead GluA1) were identified by eGFP expression and compared to uninfected cells (eGFP-) in slices from the same animal. Peak AMPAR eEPSC amplitudes (pA) across increasing stimulus intensities are plotted (mean ± SEM). Rectification index is plotted as the ratio of eEPSC -70mV /eEPSC +40mV (mean ± SEM) obtained from 5-10 eEPSCs evoked at 0.1 Hz. A) Representative eEPSC traces across stimulation intensities and representative eEPSC traces at +40mV and -70mV for eGFP+ and eGFP- neurons. B) The input-output relationship of AMPAR eEPSCS illustrates decreased synaptic strength in MSNs expressing GluA1-Q582E (n=6 cells). Bonferroni *post hoc p* <0.001 for peak eEPSC amplitude compared to non-GFP controls (n=5 cells) at 3X stimulation. C) Decreased rectification index in MSNs expressing GluA1-Q582E (n=3 cells) indicates reduced CP-AMPAR contribution. *p* <0.025 for rectification index compared to non-GFP controls (n=5).

FIGURE 3.3

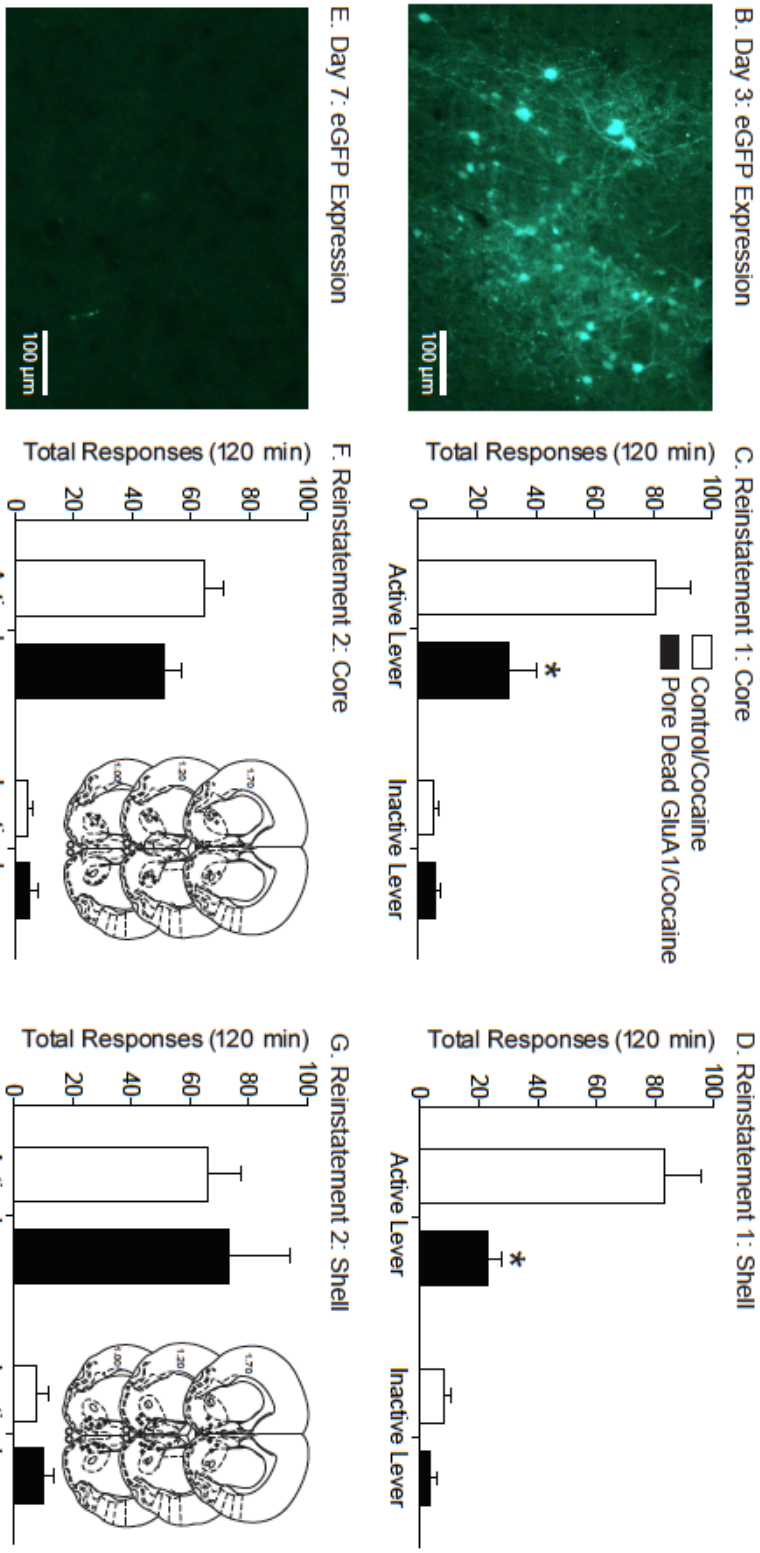
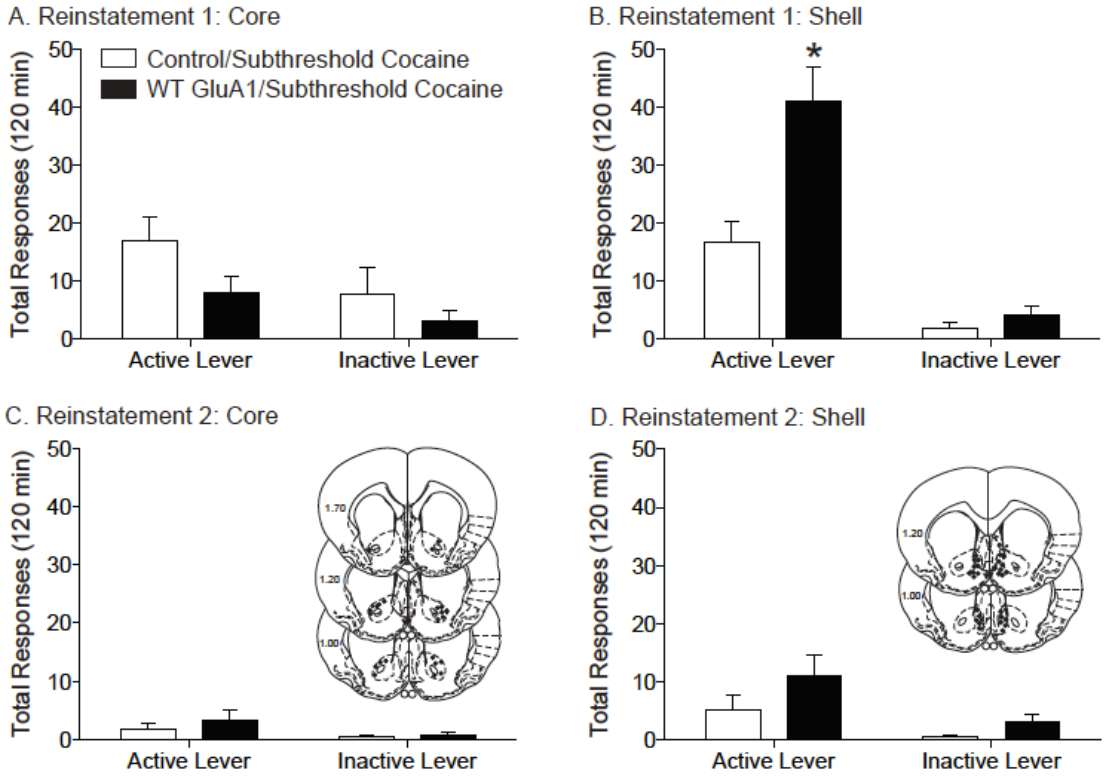

B. Day 3: eGFP Expression
100 µm
C. Reinstatement 1: Core
Control/Cocaine
Pore Dead GluA1/Cocaine
Total Responses (120 min)
Active Lever
Inactive Lever
*
D. Reinstatement 1: Shell
E. Day 7: eGFP Expression
100 µm
F. Reinstatement 2: Core
G. Reinstatement 2: Shell

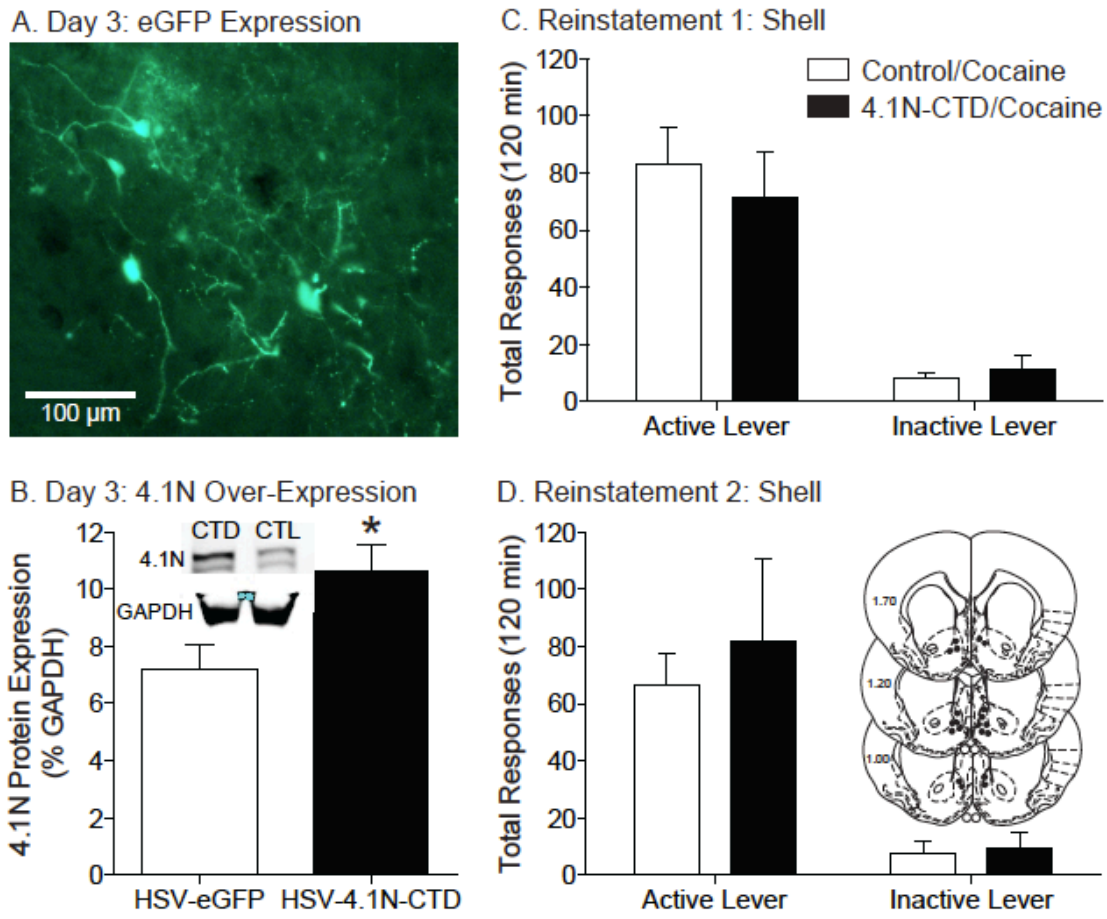
Figure 3.3: Over-expression of pore-dead GluA1-Q582E in the nucleus accumbens core or shell reversibly attenuates cocaine priming-induced reinstatement. The data are expressed as the mean (± SEM) lever responding on the active and inactive levers during the 2-hour reinstatement test sessions. A) Schematic of experimental paradigm. B) Representative image of peak eGFP fluorescence in accumbens 3 days following GluA1-Q582E infusion. C) Total lever responding following intra-core microinfusion of HSV eGFP (n=9) or HSV GluA1-Q582E (n=8) 72 hours prior to a systemic injection of cocaine (10 mg/kg, i.p.). Bonferroni *post hoc* $p<0.05$ for active lever responding between eGFP- and GluA1-Q582E-treated animals. D) Total lever responding following intra-shell microinfusion of HSV eGFP (n=13) or HSV GluA1-Q582E (n=8) 72 hours prior to a systemic injection of cocaine (10 mg/kg, i.p.). Bonferroni *post hoc* $p<0.05$ for active lever responding between eGFP- and GluA1-Q582E-treated animals. E) Representative image of absent eGFP fluorescence 7 days following GluA1-Q582E infusion due to viral dormancy. F) Total lever responding following intra-core microinfusion of HSV eGFP (n=5) or HSV GluA1-Q582E (n=6) 1 week prior to a systemic injection of cocaine (10 mg/kg, i.p.) with cannula placements for core microinfusions. The numbers indicate mm anterior to bregma according to Paxinos and Watson (1997). G) Total lever responding following intra-shell microinfusion of HSV eGFP (n=7) or HSV GluA1-Q582E (n=6) 1 week prior to a systemic injection of cocaine (10 mg/kg, i.p.) with cannula placements for shell microinfusions.

FIGURE 3.4



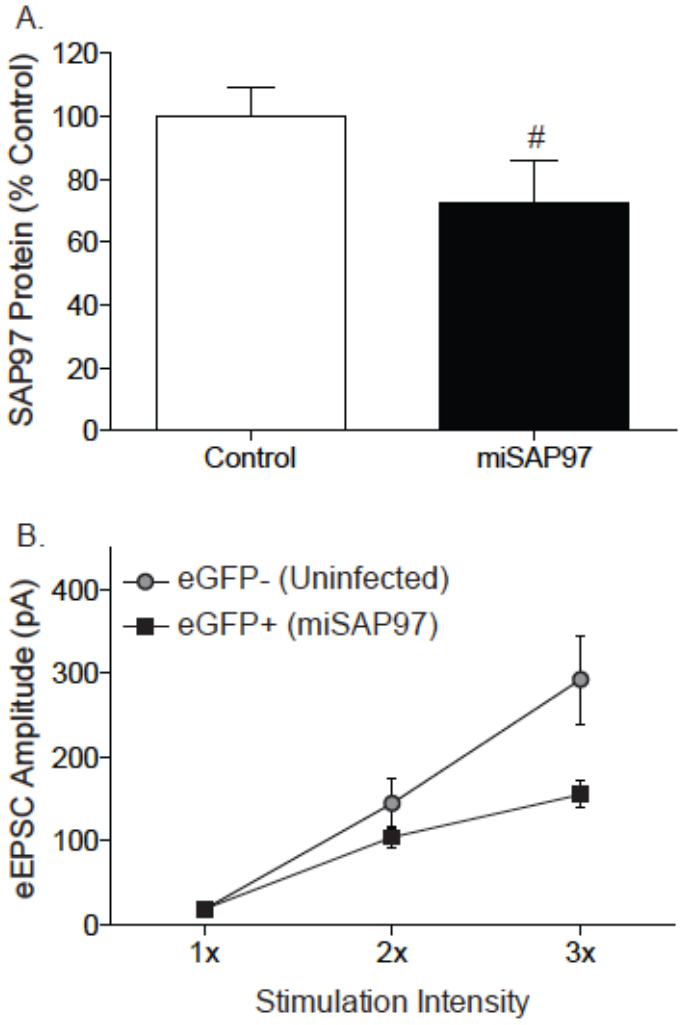
Over-expression of GluA1-WT in the nucleus accumbens shell, but not the core, promotes cocaine priming-induced reinstatement. The data are expressed as the mean (± SEM) lever responding on the active and inactive levers during the 2-hour reinstatement test sessions. A) Total lever responding following intra-core microinfusion of HSV eGFP (n=7) or HSV GluA1-WT (n=8) 72 hours prior to a systemic injection of cocaine (5 mg/kg, i.p.). B) Total lever responding following intra-shell microinfusion of HSV eGFP (n=8) or HSV GluA1-WT (n=9) 72 hours prior to a systemic injection of cocaine (5 mg/kg, i.p.). Bonferroni *post hoc* $p<0.05$ for active lever responding between eGFP- and GluA1-WT-treated animals. B) Total lever responding following intra-core microinfusion of HSV eGFP (n=7) or HSV GluA1-WT (n=8) 1 week prior to a systemic injection of cocaine (5 mg/kg, i.p.) with cannula placements for intra-core microinfusions. The numbers indicate mm anterior to bregma according to Paxinos and Watson (1997). D) Total lever responding following intra-shell microinfusion of HSV eGFP (n=7) or HSV GluA1-WT (n=9) 1 week prior to a systemic injection of cocaine (5 mg/kg, i.p.) with cannula placements for intra-shell microinfusions.

FIGURE 3.5



Over-expression of 4.1N-CTD in the nucleus accumbens shell has no effect on cocaine priming-induced reinstatement. The data are expressed as the mean (± SEM) lever responding on the active and inactive levers during the 2-hour reinstatement test sessions. A) Representative image of peak eGFP fluorescence in accumbens 3 days following 4.1N-CTD infusion. B) Western blot quantification (mean ± SEM) of 4.1N protein expression 3 days following eGFP infusion (n=10) or 4.1N-CTD infusion (n=10) into the accumbens shell and representative bands. 4.1N-CTD significantly increased 4.1N protein expression compared to eGFP control (p<0.0148). C) Total lever responding following intra-shell microinfusion of HSV eGFP (n=13) or HSV 4.1N-CTD (n=8) 72 hours prior to a systemic injection of cocaine (10 mg/kg, i.p.). D) Total lever responding following intra-core microinfusion of HSV eGFP (n=7) or HSV 4.1N-CTD (n=5) 1 week prior to a systemic injection of cocaine (10 mg/kg, i.p.) with cannula placements for intra-shell microinfusions. The numbers indicate mm anterior to bregma according to Paxinos and Watson (1997).

FIGURE 3.6



Preliminary characterization of HSV miSAP97 indicates knock down of SAP97 protein with no effect on accumbens MSN synaptic strength. For *in vivo* knockdown experiments, protein expression levels of SAP97 in total homogenate 4 days following viral microinfusion (2 µl/side) into the accumbens core and shell were measured using quantitative western blot (mean ± SEM). A) Microinfusion of miSAP97 (n=6) reduces SAP97 protein expression compared to Scramble control (n=6) injections (p<0.1198). B) Infected cells (eGFP+ miSAP97) were identified by eGFP expression and compared to uninfected cells (eGFP-) in slices from the same animal. Peak AMPAR eEPSC amplitudes (pA) across increasing stimulus intensities are plotted (mean ± SEM) in B. Preliminary analysis of the input-output relationship of AMPAR eEPSCS illustrates unchanged synaptic strength in MSNs expressing miSAP97 (n=3 cells).

Figure 3.7

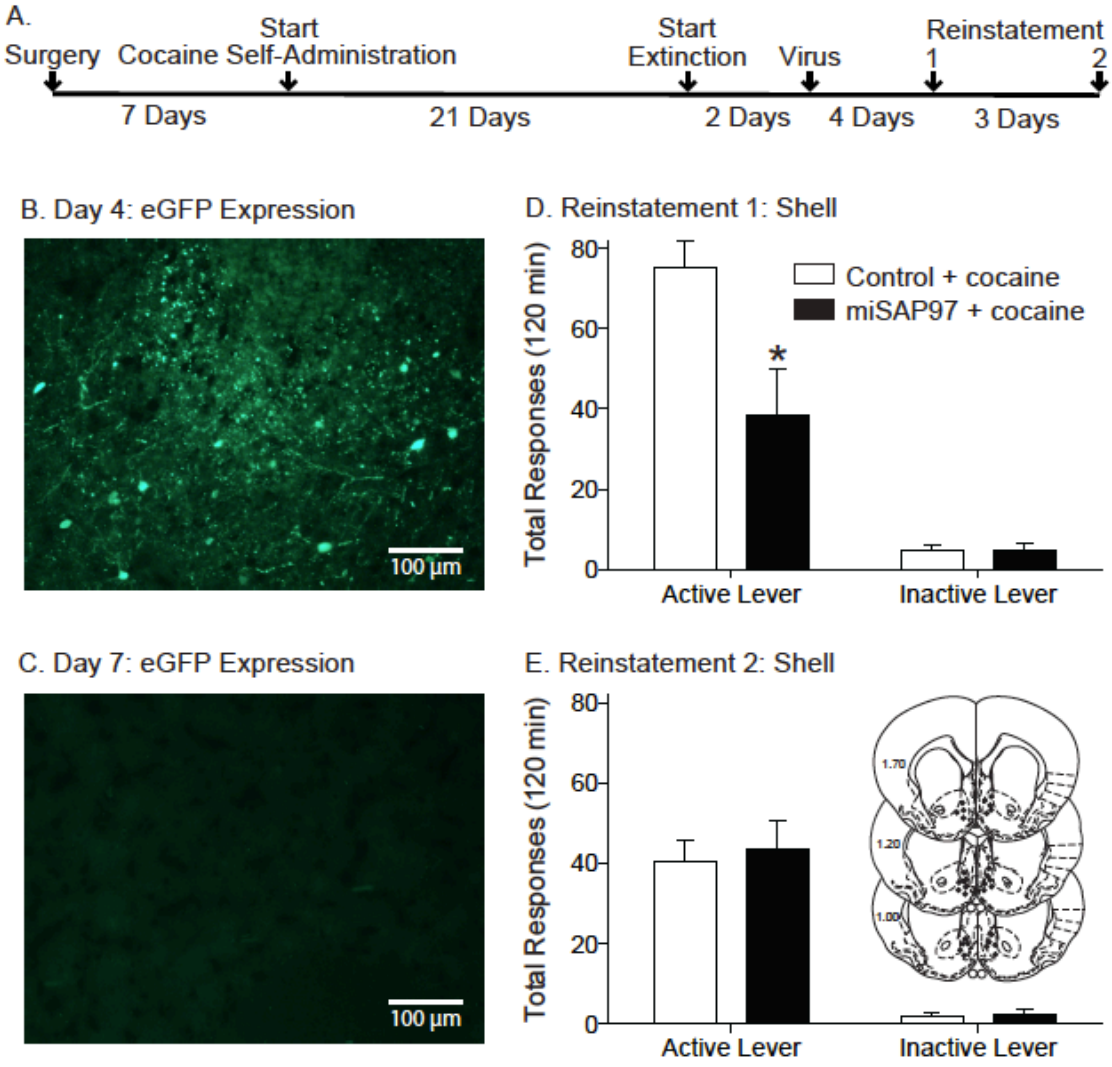

A.
Surgery
Start Cocaine Self-Administration
Start Extinction
Virus
Reinstatement 1
2
7 Days
21 Days
2 Days
4 Days
3 Days
B. Day 4: eGFP Expression
100 µm
C. Day 7: eGFP Expression
100 µm
D. Reinstatement 1: Shell
Control + cocaine
miSAP97 + cocaine
Total Responses (120 min)
80
60
40
20
0
*
Active Lever
Inactive Lever
E. Reinstatement 2: Shell
Total Responses (120 min)
80
60
40
20
0
1.70
1.20
1.00
Active Lever
Inactive Lever

Figure 3.7: HSV miSAP97-mediated redution of SAP97 protein expression in the accumbens shell attenuates cocaine priming-induced reinstatement. The data are expressed as the mean (± SEM) lever responding on the active and inactive levers during the 2-hour reinstatement test sessions. A) Schematic of experimental paradigm. B) Representative image of peak eGFP fluorescence in accumbens 3 days following miSAP97 infusion. C) Representative image of absent eGFP fluorescence 7 days following miSAP97 infusion due to viral dormancy. D) Total lever responding following intra-shell microinfusion of HSV Scramble (n=9) or HSV miSAP97 (n=9) 4 days prior to a systemic injection of cocaine (10 mg/kg, i.p.). $p<0.05$ for active lever responding between Scramble- and miSAP97-treated animals. E) Total lever responding following intra-shell microinfusion of HSV Scramble (n=7) or HSV miSAP97 (n=6) 1 week prior to a systemic injection of cocaine (10 mg/kg, i.p.) with cannula placements for intra-shell microinfusions. The numbers indicate mm anterior to bregma according to Paxinos and Watson (1997).

CHAPTER FOUR

ENHANCED ANXIETY IN THE MALE OFFSPRING OF SIRES THAT SELF-ADMINISTERED COCAINE

Samantha L. White[1*], Fair M. Vassoler[2*], Mathieu Wimmer[1],

Heath D. Schmidt[1] and R. Christopher Pierce[1]

1. Center for Neurobiology and Behavior

Department of Psychiatry

Perelman School of Medicine at the University of Pennsylvania

125 South 31st Street

Philadelphia, PA 19104

2. Department of Biomedical Sciences

Tufts University

200 Westboro Road

North Grafton, MA 01536

*These authors contributed equally

Abstract

We previously demonstrated that paternal cocaine exposure resulted in delayed acquisition and reduced maintenance of cocaine self-administration in male, but not female, offspring (Vassoler et al., 2013). This cocaine resistance phenotype was shown to be due to reduced cocaine reinforcing efficacy in the male offspring. It is possible, however, that enhancement of cocaine's anxiogenic effects may also contribute to reduced cocaine self-administration among male cocaine-sired rats. Here, adult male rats were allowed to self-administer cocaine (controls received saline passively) for 60 days and then were bred to naïve females. Measures of anxiety and cocaine-induced anxiogenic effects were assessed in the adult offspring. Sire cocaine experience had no effect on anxiety-like behaviors of female offspring, as measured by novelty induced hypophagia tasks. In contrast, cocaine-sired male offspring exhibited increased anxiety-like behaviors relative to saline-sired males. When challenged with an anxiogenic dose of cocaine (5 mg/kg, i.p.) measures of anxiety were enhanced in all subjects to an equal degree regardless of sire drug experience. Since anxiety and depression of often comorbid, we also assessed measures of depression. Sire cocaine experience had no effect on depression-like behaviors, as measured by the forced swim test, among male or female offspring. In a separate group of naïve littermates, select hippocampal cellular and molecular correlates of anxiety were measured. Increased mRNA and/or protein expression of tissue plasminogen activator, pro-brain derived neurotrophic factor, and CRF receptor 2 were measure in the hippocampus of cocaine-sired male offspring. Together, these data identify the presence of a baseline anxiety phenotype in the male offspring of cocaine-experienced sires that is unaltered by subsequent cocaine exposure.

Introduction

Environmental challenges to one generation can result in various behavioral and physiological adaptations in subsequent generations, which are often linked to epigenetic mechanisms (Feil and Fraga, 2012). Paternal transmittance of a cocaine-resistance phenotype to the male offspring was recently described (Vassoler et al., 2013). Adult male rats were trained to self-administer cocaine for 60 days and then bred with naïve females. Male, but not female, offspring of cocaine-exposed sires exhibited delayed acquisition and reduced maintenance of cocaine self-administration. This effect was due at least in part to increased brain-derived neurotrophic factor (BDNF) in the medial prefrontal cortex (mPFC) (Vassoler et al., 2013), which was previously shown to decrease both the reinforcing efficacy of cocaine and the reinstatement of cocaine seeking (Berglind et al., 2007; Sadri-Vakili et al., 2010). Interestingly, an epigenetic modification (acetylation) of BDNF promoters was observed in the sperm of the sires that self-administered cocaine, suggesting that cocaine-induced reprogramming of the germline was responsible for the cocaine resistance phenotype observed in the male offspring.

The delayed acquisition and decreased maintenance of cocaine self-administration among the male offspring of cocaine-experienced sires appeared to be primarily due to a decrease in the reinforcing effects of cocaine (Vassoler et al., 2013). However, it is possible that an enhancement of cocaine's aversive effects could also reduce cocaine intake in male cocaine-sired rats. It is well documented that cocaine produces both appetitive and aversive effects. This is demonstrated by the fact that rodents trained to voluntarily travel down a runway into a chamber to receive cocaine infusions exhibit avoidance and retreating behaviors at the entrance to the chamber (Ettenberg and Geist, 1991). These effects are reduced following administration of an

anxiolytic agent, which suggests that previous anxiety-like associations with the chamber in which cocaine was administered promote avoidance (Ettenberg, 2009). The appetitive-aversive effects of cocaine are time-dependent. That is, the immediate effects of cocaine are appetitive but delayed effects including agitation and anxiety are aversive (Ettenberg and Bernardi, 2007). The conditioned place preferences for cocaine are observed only when rats are exposed to a specific context immediately following cocaine administration. In contrast, when exposed to a distinct context 15 minutes following cocaine administration, rats subsequently avoid that environment (Ettenberg et al., 1999). The aversive effects of cocaine in this model were attenuated by administration of an anxiolytic drug (Ettenberg and Bernardi, 2007). Together, these results indicate that cocaine is initially reinforcing but quickly transitions to producing anxiety, which may explain instances of resilience to cocaine abuse in humans and rodents (Majewska, 2002; Kreek et al., 2009; Simmons et al., 2012).

The current experiments were designed to assess the extent to which the cocaine-resistance phenotype in male cocaine-sired rats is due to enhanced anxiogenic effects of cocaine. We assessed measures of anxiety as well as the anxiogenic effects of cocaine in the male and female offspring of cocaine-experienced sires using open field and novelty induced hypophagia (NIH) paradigms. Since anxiety and depression are often comorbid, we also used an animal model of depressive behavior (forced swim). Western blot and RT-PCR to were used to examine cellular and molecular correlates of these behaviors in select brain regions.

Materials and Methods

Animals and housing.

For the F_0 generation, we obtained adult male and female Sprague-Dawley rats (*Rattus norvegicus*) weighing 250–300 g from Taconic Laboratories (Germantown, NY). Animals remained individually housed except for 1 week of pair housing during the mating period. Food and water were available ad libitum. A 12/12 hr light/dark cycle with the lights on at 7:00 a.m. was used; all experimental procedures occurred during the light cycle.

Materials.

All self-administration experiments used Med-Associates (East Fairfield, VT) instrumentation enclosed within ventilated, sound attenuating chambers. All apparatus contained response levers, stimulus lights, food pellet dispensers and injection pumps. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Rockville, MD, USA) and dissolved in bacteriostatic 0.9% saline.

Surgery.

Researchers employed 80 mg/kg ketamine and 12 mg/kg xylazine to anesthetize the rats. After threading the catheter subcutaneously over the shoulder blade and routed to a mesh backmount platform (CamCaths, Cambridge, UK) and sutured below the skin between the shoulder blades, we positioned an indwelling silastic catheter into the right jugular vein and sutured it in place. Investigators flushed catheters daily with an antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline and sealed with plastic obturators when not in use.

F_0 Cocaine Self-Administration.

Following a 7-day recovery from surgery, we placed the F_0 males in operant chambers and randomly assigned half to lever press for cocaine infusions (0.25 mg cocaine/56 ml saline/infusion over 5 sec; the cocaine dose was not adjusted for animal weight) while the other half received non-contingent saline infusions of the same number and frequency. Rat operant training used an FR1 schedule of reinforcement. A 20-sec timeout period followed each cocaine infusion. We limited the rats to 75 infusions per daily 2-hour session. Self-administration continued for a total of 60 days.

*Breeding***.**

Twenty-four hours after the last day of self-administration, we placed F_0 male rats into a cage with naïve females. The animals remained co-housed for 7 days. Three out of 18 F_0 males continued receiving daily self-administration sessions during the breeding period. There were no differences in the behavior of the offspring so the groups were combined.

F_1 offspring of cocaine self-administering males (CocSired) or saline control males (SalSired) were littermates of animals generated in Vassoler et al., 2013 that were not exposed to cocaine. Some animals underwent food self-administration prior to behavioral testing, but were allowed at least 2 weeks with ad libitum food between tests.

Behavioral Tasks

For all behavioral tasks, offspring were weaned and group housed at P26, before being singly housed at P60. Testing occurred between P60-P100.

Novelty Induced Hypophagia (NIH)

NIH protocol was adapted from the procedures of Carr and Lucki (2010). Animals from multiple saline-sired and cocaine-sired litters were selected (n=6-10 males, n=9-10 females). 90 min prior to training and testing, food and water were removed from the animals' home cage and rats were habituated to the testing room. For 8 days of training, 5 peanut butter chips (Reese's Peanut Butter Chips, 2.257 g) were placed in a small Pyrex bowl and the bowl was placed in the rats' home cage. Rats received 15 minutes to access the peanut butter chips, after which uneaten peanut butter chips were discarded. On day 9 and day 11, the subjects were tested in their home cage. The testing sessions were identical to the training sessions. On day 10, testing was done in a novel environment. The novel cage contained a wire mesh floor and the walls were coated with 409 cleaning solution. A 60 W, 120 V light bulb was placed above each cage (8 inches), and white boards were set up on each side of the cage to reflect the light (1380 lux). For all testing sessions, animals received 15 minutes to access the peanut butter chips. Across training and testing, the latency to take the first chip from the bowl (s), number of chips eaten, and total time to finish all chips (s) was recorded. To determine the anxiogenic effects of cocaine, separate animals (naïve, saline-sired, or cocaine-sired) were randomly assigned to receive acute injections of saline, 2.5 mg/kg, or 5 mg/kg cocaine (i.p.) 30 min prior to the three test sessions. Animals were habituated to the injection on the last 3 days of training using a sham i.p. injection.

Forced Swim Test

This protocol was adapted from the procedures of Detke et al. (1995). Animals were habituated to the testing room for 90 min prior to a single, 15 min forced swim session. A clear, cylindrical, plastic chamber (12 inches in diameter, 15 inches deep) was filled to

a depth of 10 inches with water (23 C ± 1). To assess the effects of sire cocaine experience on behavior, animals from multiple saline-sired or cocaine-sired litters were selected (n=4/each). Animals were placed in the swim chamber and behavior was videotaped for 15 min. At the end of the session, rats were dried with paper towels and returned to their home cage on a heating pad to counteract hypothermic effects of the swim. Behaviors were scored by an individual blind to rat's background (i.e. saline-sired or cocaine-sired) in 5 s time bins for the first 5 min of the task. Behavioral measures included: climbing (upward directed movement of the forepaws along the side of the chamber), swimming (horizontal movement throughout the chamber), or immobility (no additional activity aside from that required to stay afloat).

Biochemical Experiments

Acute Corticosterone Response

Serum corticosterone levels were measured using an enzyme immunoassay kit according to manufacturer instructions (ADI-900-097, Enzo Life Sciences) to assess animal response to an acute stressor (single 5 min forced swim task). A clear, cylindrical, plastic chamber (12 inches in diameter, 15 inches deep) was filled to a depth of 10 inches with water (23 ± 1^0 C). Animals were placed in the swim chamber for 5 minutes, and then returned to their homecage for a 15 minute recovery period after which they were immediately decapitated. Trunk blood was collected in heparinized blood collection tubes and inverted multiple times to prevent coagulation. Blood was transferred to 1.5 mL Eppendorf tubes, centrifuged for 20 min (1.0 x g, 4^0 C), and serum was collected and stored at -80C until the immunoassay. For the assay, serum was diluted 1:100 and optical density was read at 405nm in a microplate reader (Bioteck, Synergy HT).

Western Blot

At P60, offspring not selected for behavioral tasks were sacrificed. Brains were rapidly extracted and bilateral hippocampi dissected and flash-frozen. Whole-cell hippocampal tissue was processed for Western blot as described previously (Anderson *et al*., 2008). For all samples, protein concentration was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (20 μg) were loaded and separated in 10% Tris-Glycine gels (Invitrogen) and transferred to nitrocellulose membranes using the i-Blot dry transfer system (Invitrogen). Membranes were blocked with Odyssey Blocking Buffer (928-40000), according to manufacturer instructions. Membranes were incubated overnight at 4° C with selective antibodies to: proBDNF (1:1000, Millipore), mBDNF (1:1000, Santa Cruz), CRF R2 (1:1000, Santa Cruz), tPA (1:1000, AbCam), TrkB (1:1000, Cell Signaling), or GAPDH (1:2000, Millipore). Membranes were then incubated with fluorescent secondary antibodies (1:5000, IR-dye 680 or IR-dye 800), before being imaged on an Odyssey fluorescent scanner (Licor Biosciences). GAPDH was used as a loading control and all proteins were normalized to GAPDH integrated intensity values.

RT-PCR

For quantitative real-time PCR with reverse transcription (RT–PCR) experiments, naïve SalSired and CocSired rats were handled daily for a week. Hippocampal dissections were performed and alternated between SalSired and CocSired animals. Hippocampi were flash-frozen on dry ice and stored at -80 °C. Thawed hippocampi were homogenized using a TissueRuptor (Qiagen) and RNA was purified using the RNeasy system (Qiagen) according to the manufacturer's protocol. RNA concentration and purity was quantified by NanoDrop spectrophotometry (Thermo Fisher Scientific). Generation of cDNA was carried out using Superscript II (Invitrogen).

Reactions were prepared in 96-well optical reaction plates (ABI) with optical adhesive covers (ABI) using SYBR Green PCR Master Mix (ABI). Three technical replicates were used for each animal. Reactions were carried out in the Step One Plus with an initial incubation at 95 °C for 10 min, and 40 subsequent cycles of 95 °C for 15 s, 60° C for 30 s. Primer sequences can be found in Table 1. Data were normalized to *Hprt*, *Tuba4a* and *Gapdh* before calculation of differences. Fold change was calculated from the ΔC_t values with corrections for housekeeping gene expression levels for each sample. For each sample, the ΔC_t was calculated against the mean for that gene's sample set. Next, these ΔC_t values were normalized to the ΔC_t from housekeeping genes for each sample to account for variability in cDNA input. Because C_t values are on a logarithmic scale, fold change is equal to two raised to the difference between experimental and control ΔC_t values. The data presented is the calculated mean for the biological replicates with *n* being equal to the number of biological replicates (that is, the number of rats examined).

Statistical Analyses

Novelty Induced Hypophagia Analyses

To analyze the effects of sire cocaine experience on offspring latency to feed in a novel environment, analyses were performed using a two-way ANOVA. The between-subjects factors were sire (saline-sired or cocaine-sired) and environment (home cage or novel). Bonferroni post-tests were used to establish significant difference ($p<0.05$).

To analyze the effects of sub-threshold cocaine on latency to feed in a novel environment, analyses were performed using a two-way ANOVA. The between-subjects factors were environment (home cage or novel) and challenge injection (saline, 2.5

mg/kg, or 5 mg/kg cocaine i.p.). Bonferroni post-tests were used to establish significant difference ($p<0.05$).

To analyze the effects of sub-threshold cocaine on saline- or cocaine-sired offspring latency to feed in a novel environment, analyses were performed using a two-way ANOVA. The between-subjects factors were drug challenge injection (saline or 2.5 mg/kg cocaine, i.p.) and environment (home cage or novel). Bonferroni post-tests were used to establish significant difference ($p<0.05$).

Forced Swim Test Analyses

Differences between saline-sired and cocaine-sired males during immobility, swimming, and climbing behaviors in the forced swim test were analyzed using Student's t-test (unpaired, two-tailed).

Serum Corticosterone Analyses

Quantification of serum corticosterone was performed using an enzyme immunoassay kit as described above and the MyAssays data analysis software (www.myassays.com). Corticosterone concentration (pg/mL) was calculated from optical density measurements using a 4-paramter standard curve. Analyses of serum corticosterone levels were performed using a two-way ANOVA. The between-subjects factors were sire (saline-sired or cocaine-sired) and sex (male or female). Bonferroni post-tests were used to establish significant difference ($p<0.05$)

Western Blot Data Analyses

Quantification was performed by normalizing the intensity of all bands with protein-specific antibodies to the GAPDH intensity, followed by normalizing that value to the

saline control (saline-sired male) values. Immunoblot analyses for baseline samples were performed using a t-test (unpaired, one-way, $p<0.05$). Immunoblot analyses for drug challenge samples were performed using Student's t-test (unpaired, one-tailed).

RT-PCR Data Analyses

For quantitative real-time RT–PCR experiments, *t*-tests were used to compare fold change values for each gene between cocaine- and saline-sired rats. Two-tailed *P* values are reported in Table 1.

Results

Enhanced novelty-induced hypophagia in male, but not female, cocaine-sired rats

In order to assess a measure of anxiety in cocaine-sired and saline-sired rats, latency to feed in familiar (home cage) and novel environments was assessed. Latencies (s) were analyzed using two-way mixed factors ANOVAs. Environment was the within subjects factor; sire was the between subjects factor. Analysis of the male offspring (Figure 4.1A) revealed a significant main effect of sire ($F_{1,17}$=8.44, p<0.0099), a significant main effect of environment ($F_{2,34}$=18.18, p<0.0001), and a significant sire × environment interaction ($F_{2,34}$=8.33, p<0.0011). *Post hoc* analyses indicated a significant increase in latency to feed in the novel environment in cocaine-sired relative to saline-sired males (Bonferroni, p<0.001) (Figure 4.1A). Analysis of the NIH data from female offspring (see Figure 4.1B) demonstrated a significant main effect of environment ($F_{2,34}$=34.02, p<0.0001), but no significant effect of sire (p<0.424) and no significant sire × environment interaction (p<0.496) (Figure 4.1B). Thus, novelty-induced hypophagia was observed in all groups. However, whereas siring condition had no influence on NIH in females, cocaine-sired males displayed a significantly enhanced latency to feed in a novel environment relative to saline-sired males.

Cocaine increased novelty-induced hypophagia equally in all siring conditions

We next wanted to examine whether cocaine would enhance anxiety in the NIH task. However, cocaine-induced appetite suppression was a potential confound for this experiment. Therefore, we assessed the influence of lower doses of cocaine on latency to feed in the NIH paradigm in naïve male rats (i.e. with typical siring history). Consistent with previous reports, 2.5 and 5.0 mg/kg cocaine (i.p.) had no influence on feeding in the homecage (Cooper, 1993) but dose-dependently increased latency to feed

in a novel environment (Figure 4.2). The latency to feed data were analyzed with a two-way mixed factors ANOVA. Environment was the within subjects factor; dose was the between subjects factor. Analysis of the effect of cocaine on latency to feed in naïve rats (see Figure 4.2) revealed a significant main effect of drug ($F_{2,9}$=9.64, p<0.0058), a significant main effect of environment ($F_{2,9}$=72.75, p<0.0001), and a significant drug × environment interaction ($F_{4,18}$=10.30, p<0.0002). *Post hoc* tests revealed a significant increase in latency to feed in the novel environment at 2.5 mg/kg and 5 mg/kg cocaine (i.p.) relative to saline (i.p.) (Bonferroni, p>0.01 at 2.5 mg/kg, p<0.001 at 5 mg/kg).

The effect of 2.5 mg/kg cocaine (i.p.) on NIH was assessed in saline-sired (Figure 4.3A) and cocaine-sired (Figure 4.3B) rats. Note that enhanced NIH among the male offspring of cocaine-experienced sires was replicated in that the latency to feed in the novel environment was significantly greater in cocaine-sired relative to saline sired rats [t_{17}=2.918, p<0.0096]. The data from saline-sired and cocaine-sired rats were analyzed with separate mixed-factors ANOVAs (environment=within subjects, saline-cocaine=between subjects). Analysis of the saline-sired data showed a significant main effect of drug treatment ($F_{1,13}$=4.782, p<0.0476), a significant main effect of environment ($F_{2,26}$=14.63, p<0.0001), and a significant treatment × environment interaction ($F_{2,26}$=5.097, p<0.0136). *Post hoc* tests revealed a significant difference between groups on Day 2 (Bonferroni, p<0.01) indicating that cocaine increased NIH in saline-sired rats (Figure 4.3A). Analysis of the cocaine-sired data pre-exposure to 2.5 mg/kg cocaine (i.p.) on cocaine-sired offspring latency to feed in a novel environment revealed a significant effect of environment ($F_{2,28}$=16.20, p<0.0001), but no significant effect of drug treatment (p<0.9737) and no significant treatment × environment interaction (p<0.9878). These results indicate that cocaine did not further enhance NIH in cocaine-sired rats (see Figure 4.3B).

Sire cocaine experience has no effect on immobility, swimming, or climbing behaviors in the forced swim test.

Because anxiety and depression are often comorbid, we also assessed behavior in a model of depression (forced swim test). Forced swim behaviors (immobility, swimming, or climbing) were analyzed using a two-way ANOVA with siring condition as the between subjects factor and behaviors as the within subjects factor (see Figure 4.4). Analysis revealed no significant effect of sire (p<1.00), a significant main effect of behavior ($F_{2,18}$=32.54, p<0.0001), and a significant sire × behavior interaction ($F_{2,18}$=3.62, p=0.0477). However, *post hoc* tests revealed no significant effect of sire on any behavior (immobility, swimming, or climbing).

Sire cocaine experience has no effect on serum corticosterone response 15 minutes after a 5 minute forced swim session.

To assess differences in HPA axis activation that might underlie the expression this behavioral phenotype, serum corticosterone was measured after acute swim stress. Serum corticosterone concentration (pg/mL, mean±SEM) for saline-sired and cocaine-sired offspring is plotted in Figure 4.5. Corticosterone concentrations were analyzed using a two-way ANOVA, which revealed a significant main effect of sex ($F_{1,20}$=66.49, p=0.0001), but no significant effect of sire (p=0.9683) and no significant sex × sire interaction (p=0.5900).

Increased mRNA and/or protein expression of various cellular and molecular correlates of anxiety in the hippocampus of male cocaine-sired rats.

The hippocampus modulates the expression of stress and anxiety via a variety of cellular and molecular correlates (McEwen et al., 2012). We use quantitative PCR to assess

hippocampal mRNA expression levels in male saline- and cocaine-sired rats (Table 4.1). The fold change for select transcripts is plotted in Figure 4.6A. Fold change was analyzed using a t-test (unpaired, two-tailed). There was a significant increase in *Crhr2* mRNA in cocaine-sired rats relative to saline-sired controls ($t(11)=2.553$, $p<0.05$). Additionally, there was a trend towards an increase in *Plat* mRNA in cocaine-sired rats ($t(11)=1.885$, $p=0.0862$). There were no significant differences in expression of *Bdnf* ($p=0.6413$), *Crhr1* ($p=0.8434$), or *Ntrk2* ($p=0.5704$) mRNA transcripts (Figure 4.6A).

We next measured hippocampal protein expression levels of saline- and cocaine-sired offspring using Western blot. The average fluorescent intensity for select proteins in the hippocampus, expressed as percent change from control, is plotted in Figure 4.6B. Percentages were analyzed using a t-test (unpaired, two-tailed). Analyses of sire cocaine exposure revealed an increase in expression of CRF receptor 2 ($t(12)=3.285$, $p<0.0065$), and proBDNF ($t(12)=1.964$, $p<0.05$) in cocaine-sired males. There was a trend towards increased protein expression of tPA ($t(8)=2.184$, $p<0.0605$) and TrkB receptor ($t(12)=2.079$, $p=0.0597$) in cocaine-sired males. There was no significant difference in mature BDNF ($p=0.7325$) protein expression (Figure 4.6B).

Discussion

We previously showed that sire cocaine experience reduces the acquisition and maintenance of cocaine self-administration in male, but not female, F_1 offspring (Vassoler et al., 2013). The current experiments extended this work to investigate measures of anxiety and depression in the male and female offspring of sires that self-administered cocaine. There were no differences among groups in a model of depression. However, the present findings of increased latency to feed in a novel environment indicated an anxiety-like behavioral phenotype among male, but not female, offspring of cocaine-experienced sires. Using the NIH paradigm we also assessed the anxiogenic effects of cocaine, which increased the latency to feed to a similar extent in all groups. This result indicates that enhancement of the aversive effects of cocaine does not appear to contribute to the reduced self-administration of cocaine in male cocaine-sired rats as previously described, but could have implications for aspects of emotional regulation (Vassoler et al., 2013).

Parental Drug Use and Offspring Emotional Regulation

A growing body of evidence suggests that parental drug exposure enhances anxiety-like or depressive-like behaviors in offspring. In mice, offspring of alcohol-consuming sires demonstrate increased immobility during the forced swim test that is rescued by a single dose of imipramine or propranolol, suggesting an increased anxiety-like phenotype behind the altered swim behavior (Abel and Bilitzke, 1990). Offspring from dams exposed to nicotine throughout pregnancy exhibit increased anxiety- and depressive-like behaviors that are correlated with impairments in hippocampal glutamatergic transmission (Parameshwaran et al., 2012). Perinatal exposure to the cannabinoid

receptor ligand in cannabis increases pup ultrasonic vocalizations and adult anxiety-like behaviors of offspring (Trezza et al., 2008).

The consequence of parental cocaine use on offspring emotionality has been minimally investigated. Mouse sires that experienced experimenter-delivered cocaine produced offspring with increased immobility in the tail suspension test, a model of depression, but no evidence of increased anxiety-like behavior in the elevated plus maze (Killinger et al., 2012). Conversely, prenatal cocaine exposure in rats increases anxiety-like behaviors in the elevated plus maze and open field in male offspring to a greater degree than female offspring (Salas-Ramirez et al., 2010). In the open field task, both male and female offspring with prenatal exposure to cocaine exhibit increased latency to enter the center of the arena, an indicator of enhanced anxiety (Sithisarn et al., 2011). Our study expands upon this literature by illustrating that paternal cocaine self-administration selectively increased the expression of anxiety-like behaviors in drug-naïve male offspring in the novelty induced hypophagia tasks, without also enhancing depressive-like behaviors. Additionally, acute cocaine exposure (i.p.) further increased latency to feed in saline-sired males only, suggesting impaired processing of anxiogenic stimuli in cocaine-sired males.

Cocaine Exposure and Neurochemical Correlates of Anxiety

Cocaine exposure also modulates stress hormones and associated receptors in the hippocampus and the hypothalamic-pituitary-adrenal (HPA) axis. Typically, the hippocampus inhibits the HPA axis, and activation of the HPA axis by cocaine promotes the release of corticotropin releasing factor (CRF) and corticosterone, both of which act on receptors in the hippocampus (Herman et al., 2005; Armario, 2010). In mice, a single episode of restraint stress activates the HPA axis and increases the expression of CRF

receptor 2 (CRF R2) mRNA in the hippocampus (Greetfeld et al., 2009). Prenatal cocaine exposure increases baseline corticosterone and enhances activation of the HPA axis in response to a CRF challenge injection in male offspring (Sithisarn et al., 2011). During cocaine withdrawal, increased CRF release in limbic and stress systems is associated with enhanced anxiety-like behaviors, which can be attenuated with CRF receptor antagonists (Basso et al., 1999; Sarnyai et al., 2001). CRF R2 activity can be both anxiogenic and anxiolytic, and a CRF R2 antagonist decreases cocaine withdrawal-induced enhancement of LTP in hippocampal slices (Bale and Vale, 2004; Guan et al., 2009). We found increased expression of CRF R2 mRNA and protein in the hippocampus of cocaine-sired male offspring, with no change of CRF R1 mRNA. Collectively, these data suggest that CRF R2-mediated hippocampal activity in cocaine-sired males is increased, potentially resulting in dysregulation of the HPA axis, and thus heightening anxiety in these animals. Interestingly, offspring serum corticosterone response 15 minutes after an acute stressor was unaffected by sire cocaine experience in our experiments. However, it is possible that a more full assessment of HPA axis activation across multiple time points would expose sire-dependent differences.

Hippocampal brain-derived neurotrophic factor (BDNF) expression regulates neurogenesis, LTP, and anxiety-like phenotypes, and is modulated by cocaine exposure. Mature (m) BDNF is produced when tissue plasminogen activator (tPA) cleaves the precursor protein, proBDNF, processing which is necessary for the expression of hippocampal neurogenesis and LTP via signaling through TrkB receptors (Pang et al., 2004; Lu et al., 2005; Greenberg et al., 2009). tPA is secreted from axon terminals into the extracellular space during neuronal activation (Pang and Lu, 2004). Systemic injection of mBDNF increases hippocampal BDNF expression and neurogenesis while decreasing anxiety- and depressive-like behaviors (Schmidt and Duman, 2010).

Withdrawal following repeat experimenter-administered cocaine increases the expression of total BDNF mRNA in the hippocampus and depressive-like behaviors (Filip et al., 2006). Conversely, prenatal exposure to cocaine reduces baseline expression and depolarization-evoked release of BDNF in offspring hippocampi (Yan et al., 2004). Collectively, these findings illustrate that the processing and signaling of BDNF in the hippocampus modulates the expression of anxiety and is sensitive to cocaine exposure.

In naïve cocaine-sired male offspring, we found increased expression of proBDNF protein in the hippocampus and no change in expression of mBDNF protein or total BDNF mRNA. Hippocampal proBDNF binding to p75 neurotrophin receptor (NTR), a member of the cell death pathway, increases LTD and spine atrophy, resulting in an attenuation of hippocampal-HPA control during acute stress exposure (Martinowich et al., 2007). Though it did not reach significance, the increase in TrkB receptor protein expression in cocaine-sired males may represent a compensatory adaptation to dysregulated BDNF signaling. Further, we illustrated trends towards increased expression of tPA mRNA and protein in the hippocampus of cocaine-sired males at baseline. However, this effect is not associated with increased mBDNF protein expression in cocaine-sired males, suggesting that the conversion of pro-BDNF to mBDNF by tPA is disrupted. Given that acute cocaine did not enhance the anxiety phenotype of cocaine-sired offspring, it is possible that hippocampal activation by acute cocaine challenge promotes the secretion of greater levels of tPA than in saline-sired offspring. This might result in higher expression of hippocampal mBDNF protein, the signaling through which creates a protective effect against the anxiogenic effects of cocaine. Collectively, these results suggest dysregulated BDNF processing and signaling in the hippocampus of cocaine-sired males likely contributes to the expression of anxiety-like behaviors.

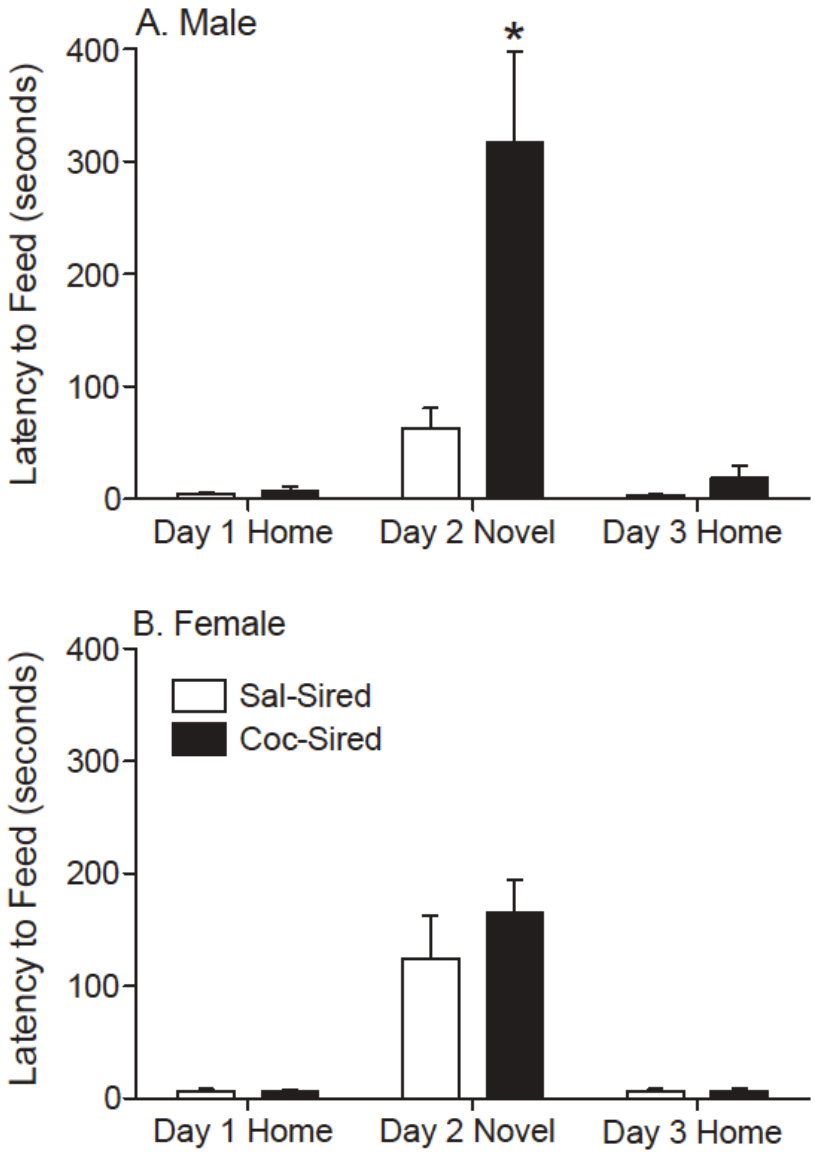
In conclusion, our findings indicated that paternal cocaine self-administration caused increased anxiety-like behaviors in male, but not in female offspring. Cocaine-sired males do not exhibit depressive-like behaviors, nor do they show altered corticosterone levels relative to saline-sired males in response to an acute stressor. Notably, acute exposure to cocaine followed by a 30-minute delay enhanced latency to feed in saline-sired, but not cocaine-sired males. We observed increases in mRNA and/or protein expression of CRF R2, tPA, TrkB, and proBDNF, in the hippocampus of cocaine-sired males. Altered signaling through CRF and BDNF pathways is correlated with anxiety-like phenotypes and dysfunction of hippocampal-HPA axis control. Thus, sire cocaine experience promotes a sex-specific, anxiety-like phenotype in offspring, potentially via modifications to proteins in the hippocampus known to influence mood regulation.

TABLE 4.1

Gene	Primer Sequences		Fold change (± SEM)	*P* value
	Forward	Reverse		
Bdnf	CCATAAGGACGCGGACTTGTAC	AGACATGTTTGCGGCATCCAGG	1.05 (± 0.07)	0.6423
Crhr1	CTGAACCCTGTGTCCACCTC	CACTCCCGGTAGCCATTGTT	1.05 (± 0.15)	0.8434
Crhr2	CAAGTACAACACGACCCGGA	TGATGATGAGGGCGATTCGG	2.40 (± 0.68)	0.0268*
Plat	GCGGCCTGAGGCAATACAAA	GAACTGATCAGCACCCCTCC	1.35 (± 0.17)	0.0862#
5htr1a	CTGTTTATCGCCCTGGATG	ATGAGCCAAGTGAGCGAGAT	1.03 (± 0.13)	0.8742
Ntrk2	GCTCTGACAGTGTTGACAGGAC	AGAAAGAGACAATGCCAGAAGC	1.09 (± 0.11)	0.5704
Hprt	GTCAAGCAGTACAGCCCCAA	TGGCCACATCAACAGGACTC	housekeeper	
Tuba4a	AGGCTCGAGAGGATATGGCT	AACACAGTGAACAGGGCTCC	housekeeper	
Gapdh	AAGATTGTCAGCAATGCATCC	ACTGTGGTCATGAGCCCTTC	housekeeper	

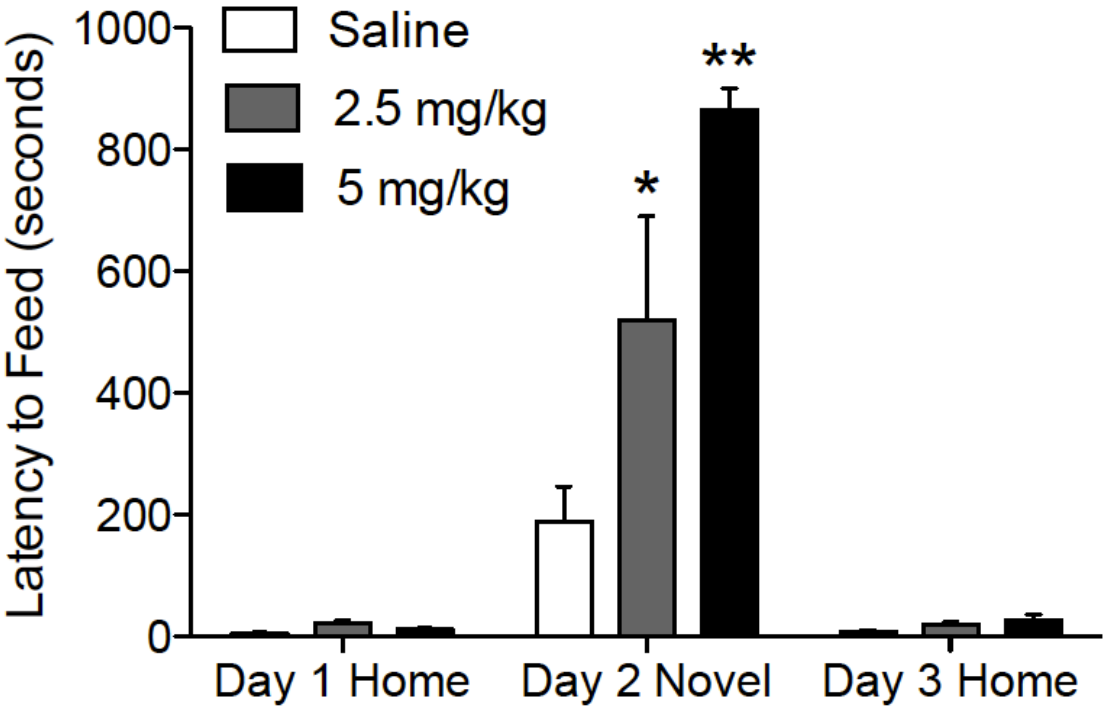
Male offspring mRNA expression levels in hippocampus. Sequence summary and statistics of measured mRNA transcripts from the hippocampus of saline-sired (n=4-5) and cocaine-sired (n=8) male offspring. Fold change of mRNA expression levels (mean ± SEM) were analyzed using a two-tailed t-Test. * indicates significant difference due to sire (p<0.05). # indicates significant trend due to sire (p<0.10).

FIGURE 4.1



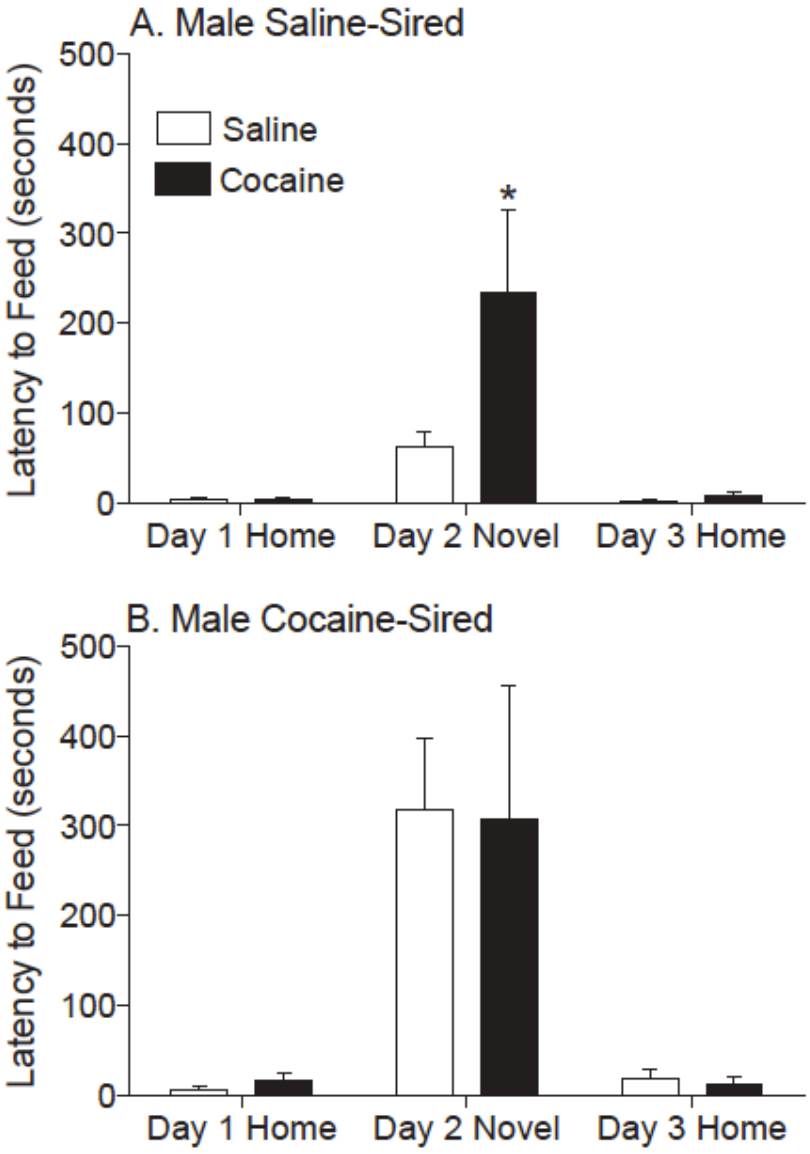
Cocaine-sired male offspring increased anxiety-like behavior. Data are expressed as latency (s, mean ± SEM) to ingest peanut butter chips in a familiar (home cage) or novel environment during a 15-minute test session. A) Cocaine-sired male offspring (n=10) have significantly increased latencies to feed compared to saline-sired male offspring (n=9). Bonferroni *post hoc* $p<0.001$ for cocaine-sired vs. saline-sired latency in the novel environment. B) There is no difference between cocaine-sired (n=10) and saline-sired (n=9) female offspring latency to feed in the novel environment. All animals displayed increased latency to feed in the novel environment (significant main effect of environment for males and females, $p<0.0001$).

FIGURE 4.2



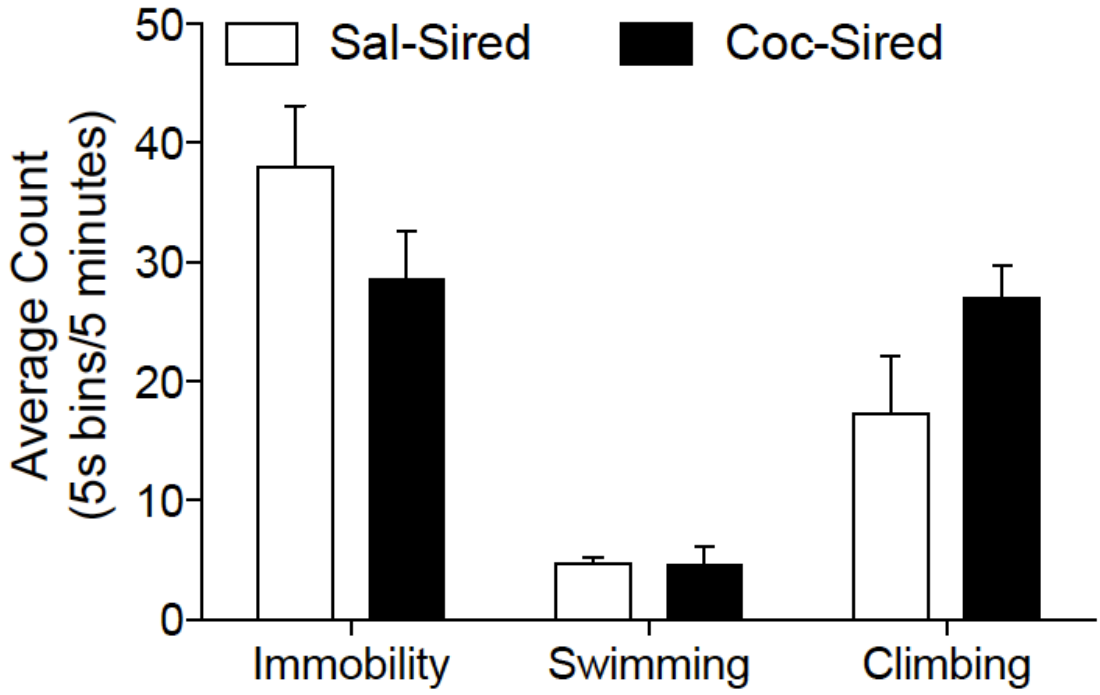
Acute cocaine increases latency to feed in a novel environment. Data are expressed as latency (s, mean ± SEM) to ingest peanut butter chips in a familiar (home cage) or novel environment during a 15-minute test session. Animals received 0 (n=4), 2.5 (n=4), or 5 mg/kg (n=4) cocaine (i.p.) 30 minutes prior to all test sessions. Cocaine dose-dependently increased latency to feed in the novel environment, but not the homecage. Bonferroni *post hoc* $p<0.01$ for 2.5 mg/kg and $p<0.001$ for 5 mg/kg relative to 0 mg/kg cocaine (i.p.) in the novel environment.

FIGURE 4.3



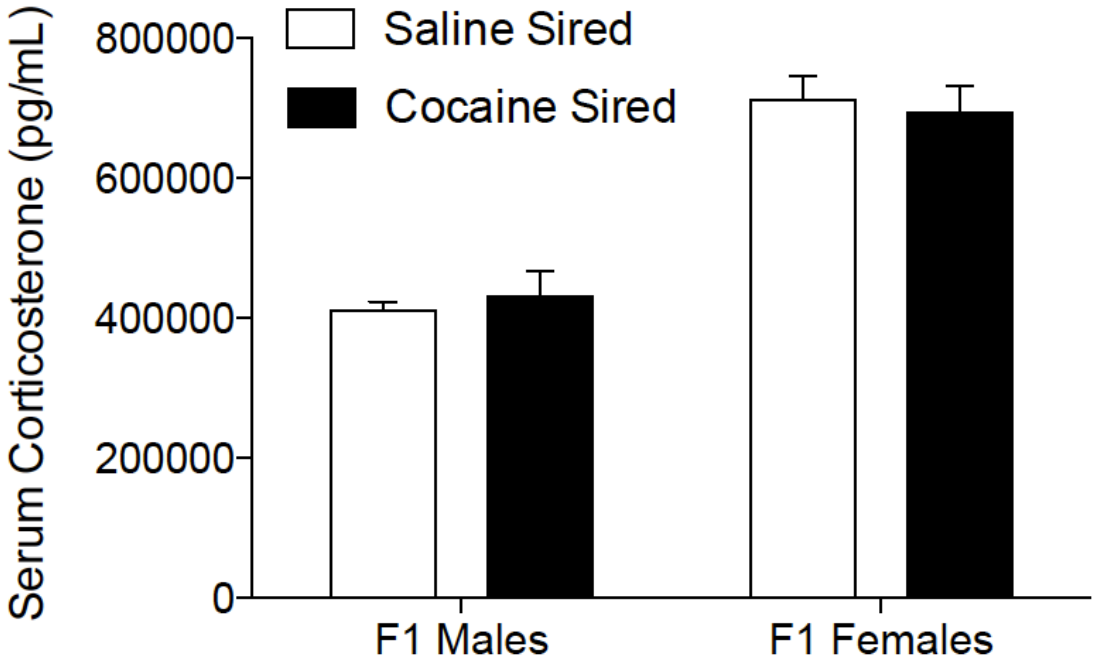
Acute cocaine increases latency to feed equally regardless of sire. Data are expressed as latency (s, mean ± SEM) to ingest peanut butter chips in a familiar (home cage) or novel environment during a 15-minute test session. Animals received the 2.5 mg/kg dose of cocaine (i.p.) 30 minutes prior to all testing sessions to avoid potential ceiling effects at the 5 mg/kg dose. A) Pre-exposure to 2.5 mg/kg cocaine (i.p.) significantly increases latency to feed in saline-sired male offspring. Bonferroni *post hoc* $p<0.01$ for 2.5 mg/kg (n=6) relative to drug-naïve (n=9) animals in the novel environment. B) Pre-exposure to 2.5 mg/kg cocaine (i.p.) did not enhance latency to feed in cocaine-sired male offspring. The novel environment increased latencies in drug-free (n=10) and pre-exposed (n=6) animals (main effect of environment $p<0.001$).

FIGURE 4.4



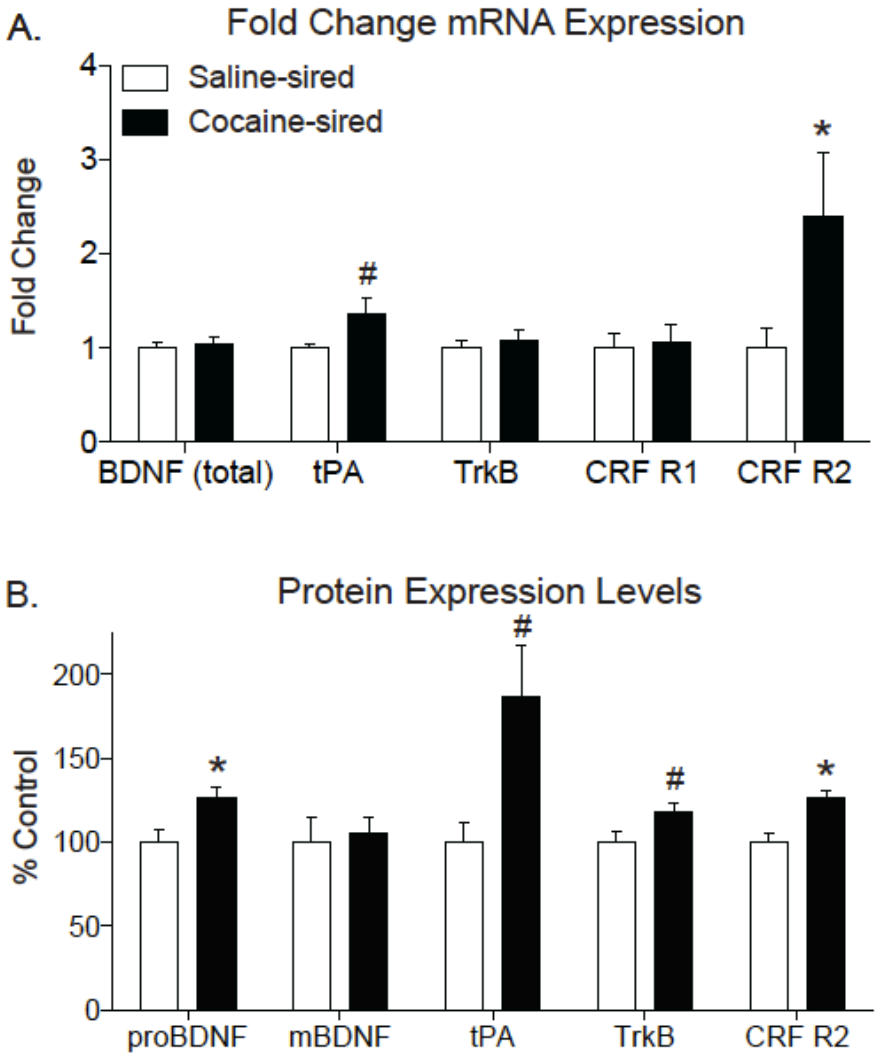
Sire cocaine experience does not influence offspring depressive-like behaviors. Data are represented as counts (mean ± SEM) of immobility, swimming, or climbing behaviors recorded every 5 seconds during a 5 minute forced swim. Cocaine-sired (n=4) and saline-sired (n=4) male offspring had no significant difference in immobility, swimming, or climbing following Bonferroni *post hoc* analysis.

FIGURE 4.5



Sired cocaine experience does not affect response to an acute stressor. Serum corticosterone concentration (pg/mL, mean ± SEM) was measured 15 minutes following a single 5-minute forced swim. Cocaine-sired (n=7) and saline-sired (n=7) female offspring had significantly increased corticosterone levels (p<0.0001) compared to cocaine-sired (n=5) and saline-sired (n=5) male offspring. There was no effect of sire cocaine experience on serum corticosterone levels.

FIGURE 4.6



Cocaine-sired male offspring have increased hippocampal expression of cellular and molecular correlates of anxiety. Select mRNA data from Table 1 are graphically represented as fold change (mean ± SEM) of select hippocampal transcripts. A) Cocaine-sired male offspring (n=8) showed a trend towards increased expression of *Plat* transcript (p<0.0863) and significantly increased expression of *Crhr2* transcript (p<0.0269) compared to saline-sired male offspring (n=4-5). There was no effect of sire on *Bdnf, Crhr1, 5htr1a,* or *Ntrk2* expression levels.

Total homogenate hippocampal protein expression levels (mean ± SEM) were quantified using western blot. B) Cocaine-sired male offspring (n=6-8) showed significantly increased expression levels of proBDNF (p<0.0293) and CRFR2 (p<0.0065), with a trend towards increased tPA (p<0.0605) and TrkB (p<0.0597) compared to saline-sired male offspring (n=4-6). There was no effect of sire on mBDNF expression levels.

CHAPTER FIVE

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Samantha L. White

Cocaine abuse in the United States represents a costly public health concern lacking efficient, lasting treatment methods. Five years after successfully completing long-term behavioral treatment for cocaine addiction, 25% of subjects report relapsing to weekly cocaine use and almost 50% of those unable to participate in long-term treatment programs succumb to relapse (Simpson et al., 2002). Preclinical work using rodent cocaine self-administration and reinstatement paradigms can help delineate the mechanisms underlying human cocaine addiction and relapse. Findings from studies using this animal model may lead to novel pharmacotherapeutic interventions that, when applied with current behavioral treatment methods, can reduce the burden of this addiction on our society. The research presented in this dissertation examined the effects of cocaine experience on brain reward circuitry during initial exposure and during cocaine priming-induced reinstatement. Additionally, these findings were expanded to include the consequences of paternal cocaine self-administration on the neurobiology and behavior of drug-naïve offspring. Together, these results have expanded our understanding of how cocaine exposure negatively influences neurological functions in both the addict and progeny, simultaneously identifying novel biochemical targets for potential treatment interventions and highlighting the pressing need to stop this disease.

Immediate Compensatory Response Following Cocaine Exposure

The data presented in chapter two described transient, compensatory biochemical adaptations in the DL striatum during the early stages of drug use. Increased phosphorylation of CaMKII (thr286) and GluA1 subunits (ser831) was illustrated in the DL, but not DM, striatum of rats following an acute, systemic injection of cocaine (10mg/kg, i.p.). Animals with cocaine self-administration experience and those exposed to cocaine priming-induced reinstatement exhibited unaltered protein expression levels relative to saline-exposed control rats. These results indicated that a tolerance to

cocaine-mediated biochemical adaptations in the DL striatum developed with extended cocaine exposure.

Altered protein expression in the DL striatum during early cocaine exposure represents previously unidentified involvement of this brain region during the initiation of cocaine seeking. Historically, reward signaling in the DL striatum occurs subsequent to transmission in the ventral striatum, a transition that requires extended cocaine exposure and the development of habit-based drug responding (Pierce and Vandeschuren, 2010; Willuhn et al., 2012). In fact, very little literature defines a role for the DL striatum following limited cocaine experience, while a large body of evidence indicates a multitude of biochemical and electrophysiological cocaine-mediated adaptations, particularly to the glutamatergic system, in the nucleus accumbens (Ferrario et al., 2010; McKee et al., 2005; Anderson et al., 2008; Edwards et al., 2007; Ortinski et al., 2012). Interestingly, transgenic mice expressing constitutively active striatal CaMKII exhibit deficits in goal-directed behavior, suggesting the relationship between drug exposure and biochemical modifications in the striatum is sensitive to time and experience (Wiltgen et al., 2007). It remains to be seen, however, how the post-translational modifications illustrated in the DL striatum might affect the development of cocaine self-administration and reinstatement. Future experiments that manipulate DL striatal CaMKII and/or GluA1 phosphorylation at initial cocaine exposure and examine the consequences the expression of cocaine priming-induced reinstatement behaviors are warranted.

The results outlined in chapter two agree with a body of work suggesting DL striatum function is not necessary during priming-induced reinstatement. Indeed, the lack of DL striatal protein expression and phosphorylation changes following cocaine

self-administration training and cocaine reinstatement suggests signaling in this region is not enlisted for these stages of cocaine experience. Processing of the rewarding effects of cocaine first occurs through projections from the VTA to the accumbens shell and core in the ventral striatum before extending into the dorsal striatum (Porrino et al., 2004). Manipulations that disrupt activity in the ventral striatum to modulate cocaine self-administration or priming-induced reinstatement have no effect on this behavior when applied to the dorsal striatum (Bari and Pierce, 2005; Vassoler et al., 2008). Additionally, I found that selective blockade of CP AMPARs in the DL striatum during cocaine priming-induced reinstatement had no effect on drug-seeking behavior despite being very effective in the nucleus accumbens (see Chapter three, Figure 3.1C). While there is some evidence of gene and protein expression changes in the DL striatum in animals with moderate cocaine self-administration experience, it is likely that the bulk of the biochemical adaptations necessary for priming-induced reinstatement do not occur in this region (Brown et al., 2011; Edwards et al., 2007; Pierce and Wolf, 2012). In fact, it is possible that processing in the ventral striatum and other areas of the reward system necessary for cocaine reinstatement underlie the reversal of modifications measured in the DL striatum after acute exposure. Reward processing in these regions can occur exclusive to the DL striatum through early stages of addiction, leaving insufficient positive feedback to maintain any modifications in the DL striatum (Bear, 2007).

Unlike reinstatement with a cocaine prime, cued reinforcement typically involves extensive training and cocaine experience, is more resistant to devaluation, and correlates with the shift from ventral to dorsal striatum signaling (Di Ciano and Everitt, 2005; Everitt et al., 2008; Pierce and Vanderschuren, 2010). Following extensive training, strengthening of excitatory synapses in DL striatal neurons is observed, indicating reliance upon this region for the signaling of habit-based behaviors (Yin et al.,

2009). Inactivation of the DL striatum after cocaine seeking has become insensitive to devaluation (i.e. habitual) produces responding sensitive to outcome devaluation, suggesting a reversal to goal-directed, ventral striatum control (Zapata et al., 2010). The short-access, 2-hour daily self-administration sessions employed in chapter two were likely insufficient to progress from goal-directed to habit-based drug seeking. It is anticipated, therefore, that additional changes to DL striatum protein expression would have been observed had animals been trained to associate drug delivery with a cue or if cocaine experience had been sufficiently extended.

The findings of chapter two point to temporary involvement of the DL striatum upon initial exposure to cocaine in spite of its established role in habit-based drug-seeking behaviors. The connection between cocaine-mediated compensatory adaptations in the DL striatum after acute exposure, adaptations identified in the reward system following additional drug experience, and the association with behavioral correlates of addiction and relapse require closer investigation. Conceivably, adaptability in the biochemical environment of the DL striatum upon initial exposure to cocaine could play a pivotal role in vulnerability to habit-based drug seeking. Consideration of how cocaine modulates the reward system prior to the point at which an individual might be considered addicted is thus required when attempting to identify therapeutic interventions.

Calcium-Permeable Glutamate Receptors During Priming-induced Reinstatement

Chapter three presented evidence that CP-AMPAR transmission in the nucleus accumbens requires interactions with GluA1-containing AMPAR accessory protein SAP97 and is necessary for cocaine priming-induced reinstatement. The CP-AMPAR antagonist Naspm or HSV vectors expressing “pore dead,” ion-impermeable GluA1

subunits (GluA1-Q582E) administered into either the core or shell of the nucleus accumbens attenuated reinstatement of cocaine-seeking behavior. However, disruption of CP-AMPAR transmission in the shell led to a specific effect on cocaine seeking, while intra-core Naspm attenuated both cocaine and food-seeking behaviors. Additionally, over-expression of functional GluA1 subunits (GluA1-WT) in the shell, but not the core, prior to a low-dose priming injection of cocaine promoted reinstatement behavior, and miRNA-mediated knock down of GluA1 accessory protein SAP97 in the shell attenuated the reinstatement of cocaine seeking. Together, this data demonstrates a role for accumbens CP-AMPARs during cocaine priming-induced reinstatement, but also implicates a protein that does not directly influence ionic or neurotransmitter concentrations as a novel target for pharmacotherapeutic manipulations.

Cocaine-mediated dopaminergic signaling differentially modulates glutamatergic transmission in the accumbens core and shell to convey information regarding reward. VTA dopaminergic projections first reach the shell, which then provides GABAergic feedback to the VTA before signaling reaches the core (Day and Carelli, 2007). During cocaine priming-induced reinstatement, activation of dopamine receptors in the shell alters AMPA receptor expression through a sequence of intracellular signaling cascades that enhances expression of GluA1-containing AMPARs to mediate behavior (Anderson et al., 2008; Famous et al., 2008; Ping et al., 2008). The primary alteration in accumbal AMPARs following cocaine experience is a shift from CI- to CP-AMPARs (for a review, see: Pierce and Wolf, 2012 and Wolf and Tseng, 2012). In chapter three, manipulations of CP-AMPAR transmission in the core and shell subregions differentially affected natural and drug reward-seeking behaviors. This provides further evidence that modulation of glutamatergic transmission in the accumbens subregions by rewarding stimuli leads to divergent neurobiological and behavioral outcomes.

Signaling in the accumbens shell is likely to mediate drug-specific reward efficacy, while the core may have a more general effect on reinforced behaviors. Previous research shows dopamine receptor antagonists infused into the shell selectively reduced cocaine self-administration, while the same antagonists attenuated both cocaine and food self-administration when microinjected into the core (Bari and Pierce, 2005). Given that dopaminergic transmission modulates glutamatergic transmission, one might predict a similar effect on behavior following application of glutamate receptor antagonists. However, injection of the broad AMPA/Kainate receptor antagonist CNQX into the core or shell selectively attenuates cocaine priming-induced reinstatement and has mixed consequences for the reinstatement of food seeking behavior (Park et al., 2002; Famous et al., 2008; Xie et al., 2012). I showed that intra-shell injection of Naspm selectively attenuated cocaine priming-induced reinstatement while intra-core microinjection also attenuated food reinstatement. These findings indicate that CP-AMPAR blockade within the shell does not lead to generalized motor deficits nor deficits in operant learning. However, transmission through accumbens core CP-AMPARs plays a more general role in the reinforcing efficacy of stimuli. This suggests that potential pharmacotherapies for addiction targeting CP-AMPARs within the entire nucleus accumbens, or the core specifically, is likely to produce side effects.

The inability of intra-core HSV GluA1-Q582E to modulate reinstatement of food-seeking behavior challenges the hypothesis that CP-AMPAR transmission in this region plays a general role in reward. One explanation is that while HSV exhibit a high infection rate in neuronal populations, fluorescent imaging provided qualitative evidence that a significant percentage of MSNs were not infected following *in vivo* injections (see Figure 3.3B; Neve et al., 2005). This might suggest that while intra-core over-expression of GluA1-Q582E attenuated cocaine reinforcement, a sufficient number of unaffected

neurons remained to allow for CP-AMPAR signaling of food reward. An alternative option is that the dominant-negative GluA1 subunits incorporate into the vast majority of AMPARs, producing a general attenuation of AMPAR transmission similar to that of the less selective antagonist, CNQX. This is supported by electrophysiological data illustrating a general reduction in AMPAR-mediated excitatory post-synaptic potentials (Figure 3.2B). As non-selective AMPAR antagonists do not alter food-seeking behavior when microinjected into the core (Kelley and Swanson, 1997; Park et al., 2002), the effects of the virus on CI-AMPARs in the core may counter the role of CP-AMPARs. Given these findings, it is likely that HSV GluA1-Q582E injection into the accumbens core did not specifically reduce transmission through CP-AMPARs to the same degree as intra-core injection of Naspm. Further, this suggests that cocaine priming-induced reinstatement is more sensitive to CP-AMPAR modulation than the reinstatement of more evolutionarily advantageous natural rewards.

Regardless of the method with which CP-AMPARs were manipulated, the involvement and necessity of functional CP-AMPAR transmission in the accumbens shell during the reinstatement of cocaine seeking was clearly illustrated in chapter three. AMPAR activation in the accumbens shell is known to elicit cocaine-seeking behavior to a greater degree than activation in the core (Ping et al., 2008). Disrupting the incorporation of GluA1-containing AMPARs into the cell surface of the accumbens shell, potentially requiring simultaneous removal of GluA2-containing AMPARs, attenuates cocaine priming-induced reinstatement (Anderson et al., 2008; Famous et al., 2008; Ping et al., 2008; Bredt and Nicoll, 2003). Similarly, my data showed attenuation of cocaine reinstatement following disrupted transmission through CP- and GluA1-containing AMPARs in the shell, in association with reduced CP-AMPAR-mediated currents. Further, over-expression of functional GluA1 subunits in the shell, but not the core,

promoted cocaine-seeking behaviors in response to a sub-threshold cocaine priming injection. This suggests that glutamate, presumably released from vmPFC efferents, binds specifically to GluA1-containing AMPARs in the shell to signal perception of reward following re-exposure to cocaine. In support of these findings, another group illustrated attenuation of D1-like dopamine receptor-mediated reinstatement of cocaine seeking following over-expression of GluA1-Q582E in the core (Hobson et al., 2013).

There is some disagreement between my findings and those of other groups who have used viral manipulations of AMPAR transmission in the accumbens subregions. One study indicates that intra-core GluA1-Q582E promotes, while GluA1-WT attenuates, cocaine priming-induced reinstatement (Bachtell et al., 2008). Methodological differences, including different self-administration and withdrawal procedures, are most likely responsible for these discrepancies. Another study finds intra-core AMPA-induced reinstatement is enhanced by GluA1-WT expression in the core, whereas intra-core expression of GluA1-WT had no effect in my experiments (Hobson et al., 2013). Hobson et al. (2013) target a more medial portion of the accumbens core where viral microinfusions are likely to overlap onto projections of the accumbens shell (Meredith et al., 2008). Thus, it is possible that unintentional GluA1-WT expression in the accumbens shell mediated the enhancement of reinstatement behavior. Alternatively, assuming viral spread is isolated to the core, it is possible that GluA1-WT over-expression increases the population of core CI-AMPARs. It is possible that synthetic AMPA-mediated activation of a large population of calcium-impermeable AMPARs sufficiently enhances MSN depolarization to alter behavioral output. This implies that cocaine-mediated glutamate release does activate the same proportion of AMPARs, which could explain the need for CP-AMPARs in order to observe changes to behavior.

On a broader note, these discrepancies underscore limitations and pitfalls of using varied reinstatement procedures to model the same human behavior.

GluA1-containing AMPAR Accessory Proteins: Novel Targets During Cocaine Reinstatement

CP-AMPARs lack the GluA2 subunit and contain GluA1 subunits, making the trafficking, stabilization, and function of GluA1-containing AMPARs in the accumbens shell an integral part of cocaine priming-induced reinstatement (Lee, 2012). Previous work indicates expression of GluA1 subunits, particularly at the cell surface of the shell, is vital to the reinstatement of cocaine-seeking behaviors (Anderson et al., 2008; Ping et al., 2008). In fact, intra-shell expression of an interfering peptide containing the GluA1 C-tail binding domains of most known accessory proteins for GluA1 prevents the trafficking of endogenous GluA1-containing AMPARs to the cell surface and attenuates cocaine priming-induced reinstatement (Anderson et al., 2008). Two accessory proteins that support GluA1-containing AMPARs are SAP97 and 4.1N, the interactions with which are likely disrupted by the GluA1 C-tail peptide (Lisman et al., 2012). In chapter three, I used alternative methods to selectively disrupt interaction of endogenous GluA1 with 4.1N or SAP97. I found that SAP97, but not 4.1N, interaction with GluA1-containing AMPARs in the acumbens shell is necessary for the cocaine priming-induced reinstatement of drug seeking. This novel finding brings to light a specific mechanism by which transmission through GluA1-containing CP-AMPARs can increase in the accumbens shell to mediate cocaine-seeking behaviors.

My initial hypothesis that protein-protein interactions between GluA1-containing AMPARs and protein 4.1N were required for cocaine reinstatement was not correct. The C-terminal domain (CTD) of 4.1N binds the membrane proximal region of GluA1

subunits to stabilize GluA1-containing AMPARs at the actin-spectrin cytoskelton, and knockdown of 4.1N reduces the insertion of GluA1 subunits into the cell surface *in vitro* (Anggono and Huganir, 2012; Lin et al., 2009). Theoretically, HSV-mediated over-expression of a dominant-negative peptide of the CTD would bind endogenous GluA1 subunits without interacting with the plasma membrane and cytoskeleton, disrupting the stabilization of GluA1-containing AMPARs, thus attenuating cocaine priming-induced reinstatement. Following accumbal microinjection, viral expression was confirmed via a 50% increase in 4.1N protein and fluorescence of GFP co-packaged with 4.1N-CTD (see Figure 3.5). Thus, it is unlikely that an absence of behavioral effect was due to methodological issues with the virus. However, due to redundancy in this system, it is possible that despite binding to 4.1N-CTD, GluA1-containing AMPARs interacted with other accessory proteins to successfully incorporate into the cell surface (Lisman et al., 2012; Jackson and Nicoll, 2011). For example, regardless of 4.1N-CTD binding, GluA1 subunits might still directly interact with SAP97 and, through SAP97 association with endogenous 4.1N, indirectly interact with endogenous 4.1N. Future experiments using co-immunoprecipitation to examine such interactions are warranted, as this would allow for unimpeded trafficking, stabilization, and function of GluA1-containing AMPARs.

In support of the idea that SAP97 may play a more critical role in the function of GluA1 subunits, we found that disrupting the association between SAP97 and GluA1-containing AMPARs attenuated cocaine priming-induced reinstatement of drug seeking. The second PDZ domain of SAP97 binds the PDZ binding motif at the extreme C-tail of GluA1 subunits while various other domains enable SAP97 to anchor at the plasma membrane (Cai et al., 2002; Zheng et al., 2011). Although some suggest this interaction is connected to the trafficking of GluA1-containing AMPARs to the cell surface (Passafaro et al., 2001; Nikandrova et al., 2010) others indicate the interaction is not

required for GluA1 subunit trafficking, but instead enhances synaptic stabilization, the formation of dendrites, and AMPAR transmission (Kim et al., 2005; Zhou et al., 2008; Howard et al., 2010). Regardless of the mechanism through which SAP97 modulates GluA1-containing AMPAR transmission, I demonstrated that knockdown of SAP97 using a micro (mi) RNA that reduces endogenous SAP97-GluA1 interactions attenuated the reinstatement of cocaine-seeking behaviors 96 hours after microinjection into the shell. This novel result identifies SAP97, not the AMPA receptor itself, as a target for potential pharmacotherapeutic interventions to prevent cocaine relapse. Modulating one of many intracellular accessory proteins instead of receptors might allow for more finely tuned adjustment of excitatory transmission that is likely to reduce side effects (like the inability to signal natural rewards).

Due to discrepancies in the literature, it was unclear how HSV miSAP97 influenced CP-AMPARs to attenuate drug-seeking behavior. Unpublished *in vitro* experiments indicate HSV miSAP97 significantly reduced SAP97 protein expression levels in numerous culture systems. Although miSAP97 appeared to knockdown endogenous SAP97 by 30% *in vivo*, these results did not reach significance, likely due to background noise from uninfected neurons. The question remained: what were the consequences for GluA1-containing AMPARs in miSAP97-infected MSNs? Surface biotinylation of accumbens from drug-naïve animals that received microinjections of HSV miSAP97 or a scrambled miRNA revealed no change in cell surface expression of GluA1 or GluA2/3 AMPAR subunits (data not shown). Preliminary electrophysiological recordings from infected and uninfected MSNs of drug-naïve animals following miSAP97 microinfusion hinted at attenuated synaptic strength and reduced inward rectification (data not shown), the hallmark of CP-AMPARs, in infected cells (Tanaka et al., 2000). Together, these findings indicate that GluA1-containing AMPARs are still able to traffic

and insert into the cell surface despite SAP97 knockdown, but without SAP97 the receptors are possibly dysfunctional, resulting in attenuated CP-AMPAR transmission. A caveat to this conclusion is that the biochemical and electrophysiological assays of HSV miSAP97 were performed in drug-naïve rodents. Given evidence that surface expression of GluA1 subunits increases in the shell during cocaine priming-induced reinstatement, examination of the receptor trafficking and electrophysiological profiles of infected neurons during cocaine reinstatement is necessary. Further, these experiments could be extended to include other scaffolding proteins known to traffic GluA1-containing AMPARs, such as stargazin, to confirm the necessity of CP-AMPAR cell surface expression to mediate the behavioral effects of cocaine (Tomita et al., 2005; Bedoukian et al., 2006; Ferrario et al., 2011b; Ferrario et al., 2011a; Jackson and Nicoll, 2011). Finally, in addition to the PDZ 2 domain that binds GluA1 subunits, SAP97 contains at least 6 additional domains and inserts known to interact with a variety of receptors and accessory proteins (Zheng et al., 2011). It is conceivable that SAP97 knockdown attenuates signaling through CP-AMPARs due to disruption of intracellular complexes at the post-synaptic density. Additional experiments involving proteomic analysis of the PSD in drug naïve and drug experience animals, as well as examination of protein expression and protein-protein interactions in the PSD following HSV miSAP97 microinfusions would clarify the mechanism through which this virus attenuates cocaine priming-induced reinstatement.

Collectively, the results of chapter three highlight manipulations of CP-AMPA receptors, which are known to mediate cocaine-seeking behavior, that could generate new pharmacotherapies to prevent relapse in a clinical setting. The circuitry involved in drug addiction, and biochemical modifications therein, shares amazing similarity with that used for learning and memory for adaptive behaviors in general (Kelley, 2004).

Accordingly, there is a need for sensitive and selective targeting of the mechanisms underlying addictive behaviors in an effort to avoid disrupting the function of natural reward systems (Hyman et al., 2006). Recent clinical trials on cocaine vaccines that produce antibodies to prevent the drug from entering the brain are promising, but face issues in maintenance of therapeutic antibody titers as well as ethical dilemmas that preclude their use in the near future (Shen et al., 2012). Short of directed brain surgery (see: Vassoler et al., 2008), most biological treatments will have an effect on the entire brain, potentially leading to undesired results. Thus, though preventing ion conductance through CP-AMPARs in the accumbens attenuated the reinstatement of cocaine seeking, the same treatment in the hippocampus might dramatically disrupt learning and memory (Lisman et al., 2012). Due to redundancy in receptor trafficking and stabilization, targeting accessory proteins, however, might allow for selective intervention in one system (the accumbens reward pathway) with fewer side effects in others (hippocampal-dependent memory) (Anggono and Huganir, 2012).

Intergenerational Consequences of Cocaine Use

Research into the treatment of cocaine addiction and relapse is largely focused on the direct effects of cocaine on the user, but a growing body of evidence suggests that there are biological and behavioral consequences for subsequent generations as well. Environmental challenges, including drug exposure, experienced by ancestors modulate the physiology and behavior of subsequent generations (Champagne, 2010; Feil and Fraga, 2012; Nestler, 2013). In humans, parental drug use greatly increases the likelihood of developmental and behavioral deficits, including drug abuse, in future generations (Merikangas et al., 1998; Kendler et al., 2007). However, it is difficult to separate the genetic and environmental factors that alter the phenotypes of the children of parents who abuse drugs like cocaine (Goldman et al., 2005). Rodent models, such

as the cocaine self-administration and reinstatement paradigm employed in this dissertation, provide controlled environmental conditions that allow for more selective investigations into the ability of cocaine to induce epigenetic changes. Delineating the inheritance of traits due to parental cocaine exposure will help to create more appropriate resources, addiction-related or otherwise, for the children of addicts.

The paradigm described in chapter four of this dissertation focuses on the potential epigenetic inheritance of neurobiological and behavioral phenotypes through a paternal lineage. The vast majority of preclinical addiction research, including that described in chapters two and three, utilized male rodents to ascertain the effects of cocaine on the brain. Females (across species) show differences in initiation and escalation of drug-seeking behavior and have differences in dopaminergic signaling in brain reward regions – all of which are modulated by ovarian hormones (Becker and Hu, 2008). Accordingly, using a paternal lineage model ensures consistency from studies of direct cocaine exposure to those examining intergenerational consequences.

From an epigenetic perspective, paternal exposure ensures the transmittance of sire cocaine experience occurs via genetic and epigenetic material contained in sperm alone (Curley et al., 2011). We have previously established that paternal cocaine self-administration prior to mating had no effect on the breeding or nurturing abilities of the drug-naïve dams (Vassoler et al., 2013). Thus, the development and maturation of first generation offspring occurred in a completely drug-free context. Although half of the genetic material of the first generation was subject to cocaine exposure, precluding any conclusions on inherited epigenetic modifications to the germline, future studies that track phenotypic differences into the second generation will provide evidence of the long-lasting, transgenerational effects of cocaine. Regardless of their context-dependent

nature, the findings of Vassoler et al. (2013) and those outlined in this dissertation provide vital evidence of the effects of cocaine outside of glutamatergic transmission in the reward system.

Paternal Cocaine Exposure Confers An Anxiety Phenotype

Expanding upon previous work illustrating a cocaine-resistance phenotype in male, but not female, offspring of cocaine-experienced sires, I identified an anxiety-like phenotype in cocaine-sired male offspring (Vassoler et al., 2013). My experiments uncovered increased anxiety-like behavior in drug-naïve cocaine-sired male offspring, suggesting intergenerational transmittance of the anxiogenic effects of cocaine. The anxiogenic properties of cocaine use are well documented across clinical and preclinical research (for review: Ettenberg, 2009; Erb, 2010). However, exposure to low-dose cocaine enhanced anxiety-like behavior of saline-sired males without exacerbating the expression of anxiety in cocaine-sired males, as measured by latency to feed in a novel environment. Our findings are in contrast to others who have described depressive-, but not anxiety-like, behaviors in the offspring of male mice exposed to non-contingent cocaine (Killinger et al., 2012). It is likely that differences in species, paradigm, and behavioral test underlie the discrepancies of particular results from our study. However, Killinger et al., came to a similar conclusion that sire cocaine experience alters the brain circuitry of mood stabilization in offspring. Together, these findings indicate impaired emotional regulation in cocaine-sired male offspring.

Although the cellular and molecular correlates to the expression of anxiety are numerous, we identified select changes to mRNA and protein expression in the hippocampus of cocaine-sired male offspring. The hippocampus is generally considered to have a role in negative feedback on stress and anxiety following HPA activation

(Herman et al., 2005; Jankord and Herman, 2008). Cocaine exposure modulates hippocampal expression and signaling of CRF and BDNF (Sarnyai et al., 1993; Yan et al., 2004; Filip et al., 2006; Guan et al., 2009). We found increased CRF R2 and tPA mRNA and protein expression and increased protein expression of proBDNF and TRkB receptor in the hippocampus of male cocaine-sired offspring. While the mechanism through which these cellular and molecular changes mediate the anxiety phenotype requires further investigation, dysregulation of these signaling pathways in the hippocampus is clear. Tying this dysregulation to epigenetic modifications of CRF and BDNF genes in the hippocampus was beyond the scope of this dissertation, but given evidence for cocaine-mediated epigenetic modifications in the sperm of sires as well as the mPFC of these offspring, future experiments using chromatin immunoprecipitation could uncover altered methylation and acetylation patterns in the hippocampus as well (Vassoler et al., 2013).

Despite evidence for connections between the serotonergic system and anxiety (Bechtholt et al., 2007; Donaldson et al., 2013), particularly with regard to cocaine exposure (Filip et al., 2010; Ettenberg et al., 2011; Pentkowski et al., 2012), initial investigations revealed no changes in cocaine-sired offspring. Given that male cocaine-sired offspring exhibit cocaine-resistant and anxiety behavioral phenotypes, it is likely that some aspects of hippocampal serotonergic signaling are altered. Future investigations into serotonergic release and the receptor populations in the hippocampus of these offspring are necessary.

There is a wealth of data connecting hippocampal BDNF and the expression of anxiety. A polymorphism in the pro-domain of BDNF (Val66Met) reduces the availability of mBDNF, potentially due to impaired processing of pro- to mBDNF by tPA, has been

correlated with increased anxiety in humans and anxiety-like behaviors, such as novelty induced hypophagia, in mice (Chen et al., 2006; Chen et al., 2008b). Similarly, increased expression of proBDNF, decreased tPA activity, and increased LTD in the hippocampus due to prenatal stress exposure increases the expression of anxiety behaviors in offspring (Rice et al., 2007; Neeley et al., 2011; Yeh et al., 2012). Conversely, tPA cleavage of proBDNF to mBDNF mediates hippocampal LTP (Pang et al., 2004) and increased expression of mBDNF in the hippocampus using viral vectors or by systemic or intra-hippocampal injection decreases novelty induced hypophagia in addition to depressive-like behaviors in rodents, likely via signaling through Trk receptors (Shirayama et al., 2002; Schmidt and Duman, 2010; Quesseveur et al., 2013). Further, viral-mediated knockdown of tPA in the dorsal hippocampus increases anxiety- and depressive-like behaviors, including latency to feed in NIH (Bahi and Dreyer, 2012). Bahi and Dreyer (2012) also show that over-expression of tPA in the hippocampus both reduces anxiety behaviors and increases expression of mBDNF protein. Thus, dysregulation of the processing and signaling of BDNF in the hippocampus disrupts mood regulation, which may provide one mechanism for the anxiety phenotype observed in the cocaine-sired male offspring.

The mechanisms through which CRF acts in the hippocampus to mediate anxiety-like behaviors are more complex. Both CRF receptors (R1 and R2) are expressed in the hippocampus, but their binding by CRF released following activation of the HPA axis does not produce the same effects (Primus et al., 1997; Bear, 2007). In some instances, stress exposure promotes anxiety by increasing hippocampal expression of CRF R1 mRNA and protein, and a CRF R1 antagonist reduces the expression of anxiety (Wang et al., 2011). Others have shown decreased expression of CRF R1 but increased expression of CRF R2 mRNA and protein in the hippocampus

following an acute restraint stress (Greetfeld et al., 2009). A selective CRF R2 antagonist administered into the ventricles reduces the expression of anxiety across a variety of behavioral tasks (Takahashi et al., 2001), but CRF R2 knockout mice typically display an impaired stress response and increased anxiety-like behaviors (Bale and Vale, 2004; Goel and Bale, 2007). Cocaine withdrawal enhances both the expression of anxiety and hippocampal LTP, but application of a selective CRF R2 antagonist decrease withdrawal-mediated enhancement of LTP (Guan et al., 2009). Interestingly, there is evidence of decreased CRF and CRF R1 expression in the hippocampus following cocaine exposure (Sarnyai et al., 2001). With regard to anxiety, the exact role of CRF receptors in the hippocampus requires further investigation, though it appears possible that at least some of the anxiogenic effects of cocaine may arise from CRF R2 signaling in the hippocampus.

Concluding Remarks

This dissertation provided evidence that cocaine exposure influences both the glutamatergic system in brain reward regions of the user and the circuitry that mediates emotional regulation in drug-naïve offspring of cocaine-experienced sires. Though much evidence indicates cocaine exerts its rewarding effects through immediate modulation of the dopaminergic and glutamatergic systems, particularly in the nucleus accumbens, the findings presented here establish novel, compensatory adaptations to AMPARs and associated proteins in the DL striatum (Wolf et al., 2003; Schmidt et al., 2005; Schmidt and Pierce, 2010; Pierce and Wolf, 2013). Given the established involvement of the DL striatum during habit-based drug seeking after extensive cocaine experience, the effects following acute exposure may indicate a more broad engagement of the mesocorticolimbic reward circuitry by cocaine that later becomes region-specific with the development of addiction (Pierce and Vanderschuren, 2010; Milton and Everitt, 2012). It

remains to be seen, however, whether disrupting the changes observed in the DL striatum would enhance or attenuate the development of addiction.

Continued cocaine experience promotes modifications of glutamatergic receptor proteins and altered excitatory transmission in the nucleus accumbens, which is particularly evident during cocaine priming-induced reinstatement (Anderson et al., 2008; Pierce and Wolf, 2013). The results of this dissertation expanded upon this knowledge, identifying a necessity for CP-AMPAR transmission that requires interaction with accessory protein SAP97 in the accumbens shell to mediate the reinstatement of cocaine seeking. Thus, therapies that manipulate accessory proteins to indirectly modulate receptor functioning may prove more fruitful in selectively combating addictive behaviors. Further, research into pharmacotherapeutic interventions for addiction should consider not only the circuitry of the relapsing addict, but also those with very limited exposure to cocaine, as glutamatergic transmission in regions that process reward is susceptible to modification at all stages of use.

Finally, although interventions focused on preventing deviant glutamatergic transmission in reward pathways are likely to address issues of addiction and relapse in the user, attention must be paid to the offspring of individuals with a history of cocaine use. Cocaine use in one generation clearly influences the behavior and physiology of the following generation, potentially via epigenetic modifications to genetic material (Nestler, 2013). The male offspring of cocaine-experienced rats exhibit altered reward processing (Vassoler et al., 2013) and, as illustrated in this dissertation, impaired emotional control. That said, the consequences of parental cocaine use are likely to extend to other types of processing, such as that for executive function and learning and memory (He et al., 2006a; Ivanov et al., 2012; Buckingham-Howes et al., 2013).

Understanding the manners and mechanisms in which parental cocaine experience affects offspring, particularly given our understanding of cocaine-mediated adaptations in the brains of addicted individuals, will improve investigations into treatment therapies for all impacted by this drug of abuse.

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