AN EXAMINATION OF D-SERINE AUGMENTATION ON THE BEHAVIORAL AND CELLULAR MECHANISMS OF COCAINE SEEKING

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology and Neuroscience Department in the Behavioral and Integrative Neuroscience Program.

Chapel Hill
2017

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ABSTRACT

Kati Lynn Healey: An Examination of D-serine Augmentation on the Behavioral and Cellular Mechanisms of Cocaine Seeking
(Under direction of Kathryn Reissner)

Withdrawal from chronic cocaine use is characterized by cellular adaptations and structural remodeling within the brain's reward circuitry, which are believed to drive persistent drug seeking and relapse. In particular, glutamatergic projections onto nucleus accumbens medium spiny neurons (MSNs) exhibit synaptic strengthening but a loss of plasticity in preclinical animal models of cocaine abuse. Recent evidence suggests that levels of D-serine, an astrocyte derived co-agonist of the NDMA receptor, are reduced in the accumbens following withdrawal from chronic non-contingent cocaine exposure, and that administration of D-serine can reverse these synaptic changes. Moreover, we have found that astrocytes in the nucleus accumbens (NAc) core make fewer synaptic connections following cocaine self-administration and extinction, suggesting reduced D-serine tone on NMDA receptors through volume diffusion. Thus, the overarching goal of this dissertation was to investigate the ability of D-serine augmentation to attenuate cocaine seeking following a cocaine plus cue-primed reinstatement event and then to characterize the mechanism by which D-serine augmentation attenuated this cocaine seeking. Using the self-administration and extinction model of cocaine abuse, the present series of experiments found that systemic and intra-accumbal D-serine augmentation attenuated cocaine seeking, and that systemic D-serine augmentation depotentiated the synapse and enhanced NMDA receptor activation in the NAc core. Interestingly, the experiments presented here conclude that there are no changes in surface protein expression of glutamatergic ionotropic subunits from the NMDA and AMPA receptors following D-serine

augmentation. Therefore, the experiments were unable to determine the direct mechanism of action by which D-serine augmentation is reducing relapse to cocaine seeking. However, it can be concluded that NMDA receptor induced internalization of AMPA receptors is not likely the mechanism of the D-serine augmentation effect. Together, these data enhance the current understanding of NMDA receptor involvement in cocaine relapse behaviors, such that NMDA receptors oppose reinstatement of cocaine seeking. We also provide a detailed characterization of NMDA and AMPA receptor expression and synaptic function after enhancing the NMDA receptor co-agonist D-serine.

ACKNOWLEDGEMENTS

My sincere gratitude to:

The members of my dissertation committee: Kathryn Reissner, Don Lysle, Regina Carrelli, Kelly Giovanello, and Sylvia Fitting. Thank you for your guidance and mentorship throughout this dissertation process.

My advisor, Kate. Thank you for all of your support throughout my time in your laboratory. I have grown so much as a researcher and a person under your tutelage. I gained invaluable experience in watching you build your laboratory from the ground up - with great enthusiasm. It's been an honor to be your first graduate student, and I know that I will bring many the qualities you taught me into the next stages of my career and, I hope, as a mentor myself.

The past and present members of the Reissner Lab: Marian Sepulveda Orengo, Ron Kim, Anze Testen, Emily Williams, Ben Wu, Francia Fang, Kara Lopez, Mahi Yetukuri, Alyson Auriemma, Nicholas Woronoff and Suzanne Sherille. Thank you for making this lab so enjoyable to be in and for your constant and enduring support during my research process. Without the encouragement of the great people in this laboratory I would never have made it this far. Also, the Fuchs lab, Rita Fuchs Lokensgard, Amy Arguello, Audrey Wells, Sierra Stringfield, Xiaohu "Tiger" Xie and Mark Presker for getting me moving on this grad school journey and continuing to encourage and support me across the country and the eastern seaboard.

Finally, my family and my friends who are family. Your constant unending support and love has helped me through the entirety of my education, especially graduate school. You were my persistent cheerleading squad, and have endured many of my practice lectures and talks (with hopefully more to come in the future) with patience and kindness.

And of course, for Athena. You all mean the world to me.

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3-PGDH	3-phosphogylcerate dehydrogenase
acsf	Artificial cerebrospinal fluid
AMPA	α-amino-3hydroxy-5-methyl-4isoxazoleproprionic acid
ANOVA	Analysis of variance
APV	(2R)-amino-5-phosphonovaleric acid
Asc1	Alanine-serine-cysteine-1 transporter-1
ASCT	Alanine/serine/cysteine/threonine transporter
DAO	D-amino acid oxidase
D2	Dopamine receptor 2
DS	D-serine
FR	Fixed ratio
GABA	γ-aminobutryric acid
GLT-1	Glutamate transporter type 1
GMS	Glycine modulatory site
HPLC	High-pressure liquid chromatography
iv	Intravenous
i.p.	Intra-peritoneal
kDa	Kilodalton
LTD	Long-term depression
LTP	Long-term potentiation
ME	Main effect
min	Minutes
mPFC	medial prefrontal cortex

MSN	Medium spiny neuron
NIDA	National Institute on Drug Abuse
NMDA	*N*-methyl-D-aspartate
NAc	Nucleus accumbens
PL	Prelimbic cortex
SB	Sodium benzoate
SE	Surface expression
SR	Serine racemase
STI	Sexual transmitted infection
T=0	Time=0 (basal)
T=1	Time=1 hour (reinstatement test)
TP	Total protein
Veh	Vehicle
VTA	Ventral tegmental area

CHAPTER 1:

General Introduction.

Cocaine Use and Abuse: Then and Now

Over 4 centuries ago (3000-2000 BC) in Ecuador is the earlies indication of coca use by humans (Van Dyke, C 1984 PMID 7043731). The first documented cultivation and use of the coca leaf was in 700 BC in the Andes (Calatayud & González, 2003). The Incas would chew coca leaves thought to be given to them by the gods, the first reported use of cocaine(Calatayud & González, 2003; Goldstein, DesLauriers, & Burda, 2009). Chewing coca leaves or brewing them in tea gives an energy boost and has long been used to treat headaches, toothaches and intestinal cramps in the Andes and other areas of South America. They also are high in calcium and can relieve altitude sickness, possibly why this tradition, started many centuries ago, is still common practice in the Andes. Native Peruvians had strict rules to chew coca only during religious ceremonies, however this changed in 1500 AD when the Spanish invaded and forced the natives to work in their silver mines. The Spanish started requiring the workers to chew coca leaves to make them easier to control and exploit in their mines.

In 1860, the main active ingredient in coca leaves was extracted by German Chemist Albert Niemann, but it wasn't until 1898 that cocaine was synthetically produced by Dr. Richard Willstatter. Shortly after Niemann improved the purification process, cocaine received a famous champion in Dr. Sigmund Freud when he was completing his doctoral work in Austria. Dr. Freud was enthusiastic about the implications of using cocaine as a numbing agent for surgeries, and even became a proponent for recreational use, using the drug himself copiously. He advocated

that cocaine could be used as a tonic to cure depression and sexual impotence and even went so far as to write a document on the "magical" substance's benefits entitled *Uber Coca* in 1884.

Across the pond, an American surgeon also became enamored with cocaine, Dr. William Stewart Halsted. He was one of the first surgeons to emphasize a strict aseptic technique during surgeries (we can thank him for surgical gloves) and was a proponent of early anesthetics. He often used himself as a test subject, and encouraged other doctors to do the same, however, a result of these experimentations was that Dr. Halsted battled addiction to cocaine, and later morphine, until the end of his life (Imber).

Parallel to these medical interests in cocaine, cocaine and opium-laced elixirs, tonics and wines became broadly used by all social classes. John Pemberton included coca leaves in his new soft drink, creating Coca-Cola in 1886. The drink had immediate popularity, and continued using the coca leaves until 1901 when they were removed due to pressure from the increasing health crises of cocaine addiction, at which time caffeine was substituted for cocaine (Karch, 2005). Icons such as Jules Verne, Thomas Edison and Sara Bernhardt advocated for *Vin Mariani*, a cocaine laced wine, in advertisements and Bernhardt's endorsement is an example of the pro-cocaine sentiment from Hollywood that influenced millions.

By 1905 it was discovered that cocaine did not need to be in a solution and that snorting the drug could give a more potent high. Within 5 years nasal damage from snorting cocaine was seen in hospitals and was reported in medical journals (Karch, 2005). In 1914, the use of cocaine was outlawed by the *Harrison Narcotics Tax Act*. However, modern criminal laws that are enforced today come from the *Controlled Substances Act* of 1970.

In 1961 an international treaty was enacted entitled the Single Convention on Narcotic Drugs requiring countries to make recreational use of cocaine and other narcotics a crime (Room & Reuter, 2012). Soon after the international treaty and the *Controlled Substances Act,* cocaine became a serious problem in the United States of America (US). Colombian drug traffickers began setting up elaborate trafficking networks in the 1970's to ship their cocaine to

the US. As a result, by 1990 drug cartels produced and exported tons of cocaine per year to the US and also to Europe and Asia. In retaliation, law enforcement targeted and dismantled the Columbian drug cartels throughout the 1990s. Today, smaller cartels remain, and as of 2008 cocaine had become the second most illegally long-distance trafficked drug in the world (UNODC, 2010).

In 2009 the NDIC reported that intercartel fighting and expanding drug markets in Europe and elsewhere has lowered cocaine availability in the US, driving a 69% increase in cocaine prices in the 3rd quarter of 2010 (STRIDE, 2010). Along with this decrease in availability and increased costs, it was also reported in 2009 by the National Survey on Drug Use and Health (NSDUH) that the rate of cocaine use among individuals 12 and older declined to 1.9% from 2.5% in 2006 (NSDUH, 2011) and that trend has continued in 2016 with prevalence of use in the last 12 months of cocaine in ages 12 or older at 1.9% (NSDUH 2016 (NSDUH, 2016).

Societal costs of cocaine abuse remain high, despite the plateau of drug use. In 2006 there were 7,475 fatal cocaine poisonings in the US, approximately 20% of all drug related deaths (SAMHSA, 2012). However, this may be a conservative estimate, as other studies have reported cocaine related deaths make up 40% of all drug related deaths, costing the US an estimated $13 billion in cocaine related premature deaths (UNODC, 2010). Further, the Drug Abuse Warning Network (DAWN) reported in 2011 that of the nearly 1.3 million emergency room visits for drug use or misuse, cocaine was present in over 500,000 of these visits (NSDUH, 2011).

Substance abuse in all classes of drugs cost the US over $700 billion annually (National Institute on Drug Abuse [NIDA], 2015), as such investment in addiction treatment should be a high priority to reduce these costs. Summarized by the NIH, assessments state that for every $1 invested in addiction treatment $5-7 can be returned in savings of drug related crimes, criminal justice cost and theft (NIDA, 2016). A modest cocaine abuse disorder medication, that would reduce 1995 cocaine intake levels by 1% was approximated in 2000 to save the US $259 million

(Cartwright, 2000), further a modest medication that could increase abstinence by 10% would have a cost benefit ratio between 1.59-5.79 (Cartwright, 2000). Accordingly, development of novel effective pharmacotherapeutics that combat addiction are necessary to offset the societal costs of substance abuse disorders. Unfortunately, however, there are currently no FDA-approved medications to combat psychostimulant use disorders.

Cocaine pharmacology and the physical effects of cocaine use

Cocaine is a Schedule II drug, indicating that it has a high abuse potential but also has legitimate medical uses, which include applications as a local anesthetic in eye, ear, nose, throat and dental surgeries. Cocaine is the second most widely abused illegal drug globally, after cannabis (Karila et al., 2014; UNODC, 2010). People abuse cocaine in two forms: soluble cocaine hydrochloride salt and insoluble cocaine base (freebase; (NIDA, 2016)). Processing cocaine hydrochloride further with ammonia or sodium bicarbonate yields cocaine freebase or crack cocaine, which can be smoked (Goldstein et al., 2009). Abusers most often use cocaine in binge patterns, escalating their dose to maintain a potent high and counteract tolerance. Unfortunately, street dealers mix cocaine with other products to increase profit. They use things such as cornstarch, talcum powder or flour or can mix with other drugs like amphetamine or fentanyl, potentially lethal combinations.

Cocaine easily crosses the blood brain barrier, allowing for the euphoric effects of the drug to set in quickly. The drug effects last for 15-30 minutes after snorting cocaine powder and the high from inhalation through smoking or other means lasts for 5-10 minutes. Physical symptoms include: fast heart rate, sweating, dilated pupils, high blood pressure or body temperature, nausea, constricted blood vessels, tremors and muscle twitches, and restlessness (NIDA, 2016). Cognitive symptoms include: alertness, extreme happiness and energy, hypersensitivity to sound, sight and touch, irritability, and paranoia ((NIDA, 2016).

Long-term effects of cocaine use can lead to loss of smell, nosebleeds, frequent runny nose, and problems swallowing in individuals who snort cocaine. Consumption of cocaine by mouth can lead to severe bowel decay due to reduced blood flow in the intestines (NIDA, 2016) and intravenous cocaine users have a higher risk of contracting Human Immunodeficiency Virus (HIV), hepatitis C, and other blood borne diseases. Use of cocaine in all forms leads to impaired judgment, which can lead to risky sexual behaviors that are associated with high risk of sexually transmitted infections (STI;(NIDA, 2016)). Other long-term effects of cocaine use are malnourishment, movement disorders (or disorders of dopamine), severe paranoia where the abuser loses touch with reality and auditory hallucinations and importantly impaired cardiovascular function and risk of heart disease (NIDA, 2016).

The chemical name for cocaine is benzoylmethylecgonine and its mechanism of action is to inhibit the reuptake of serotonin, norepinephrine and dopamine. Cocaine binds to the dopamine reuptake transporter (DAT, NET and SERT) and blocks the transporter from removing neurotransmitter, particularly dopamine, from the synapse. Accordingly, dopamine remains in the synapse longer, activating the reward pathway for an extended period of time. Prolonged use of cocaine leads to numerous neuroadaptations in the reward pathway, including disruptions of glutamate homeostasis (Kalivas, 2009) and glucose metabolism (Volkow, Wang, Fowler, Tomasi, & Telang, 2011), the former to be discussed in further detail later. These lasting neuroadaptations and high prevalence of cocaine abuse in the US and around the world, combined with the medical and socioeconomic consequences mandate a better understanding of the mechanisms responsible for reward, dependence and addiction. Developments in animal models of addiction have and continue to facilitate this endeavor, in a search for pharmacotherapies to combat psychostimulant addiction.

Modeling intravenous cocaine administration in the laboratory.

It wasn't until the 20th century that scientists began to explore a new type of learning apart from the established associative learning, stimulus-response learning. The first to study stimulus-response learning was Dr. E.L. Thorndike, who for his doctoral thesis set up puzzle boxes that hungry cats could escape from to get to food located outside the box by various behavioral responses (e.g. pulling a cord or a lever; (THORNDIKE, 1898)). These were discrete trails, because the cat could only escape the box once to consume the reward and then the trial was completed. Following Dr. Thorndike, Dr. B.F. Skinner came up with a more continuous way to study stimulus-response learning, in what is called free-operant conditioning. In these experiments animals are allowed to repeat the response over and over without constraint or removal from the apparatus until the designated end of the trial. This allows for study of continuous behavior in a way that mazes and puzzle boxes are unable to do, due to the nature of the discrete trial design.

Free-operant conditioning allows for the study of behavior in the context of the probability of future occurrences. The operant response is defined by how the behavior operates on the environment, rather than what the behavior is itself. In contrast, the instrumental response is what is necessary to produce the desired consequence or outcome. Newer models of reinforced behavior emphasize that actions of reinforcement need to be considered and understood in a broader context relating learning and memory (Hyman, 2005; N. M. White, 1996), instead of the simpler Skinnerian view that events following a response solely guide the probability of future occurrences of that response.

Simple schedules of operant conditioning can be used to elucidate mechanisms of relapse to drugs of abuse and other aspects of substance use disorders. A hallmark of addiction in the clinical population is compulsive drug seeking and relapse to drug use; these features separate addiction from recreational use. In preclinical experiments, operant training for drug delivery schedules is an example of positive reinforcement in which the drug of abuse is the reinforcer,

increasing the instrumental response to self-administer the reward. The route of self-administration is important, and in preclinical cocaine research intravenous administration is the most prevalent. An operant chamber ("Skinner box") is necessary to perform intravenous self-administration behavior. The configuration of theses boxes can be adjusted for the needs of the experiment, and most commonly have levers for responding, however other more "naturally" occurring behaviors such as nose poke (rodents) or key pecking (pigeons) can be incorporated in these boxes. They also allow for programmed events such as lights, tones and smells that can be paired with drug delivery and be used as discrete stimuli or secondary reinforcers.

The most common model of self-administration in drug literature is using a fixed-ratio (FR) schedule of drug delivery. Under this schedule the subject must perform the operant response a certain number of times to obtain the reward, and this number is fixed throughout the self-administration session. Generally, experiments use an FR1 schedule (or continuous reinforcement) where the operant response must be performed one time to receive the reinforcer (drug). There is generally a time out period (often 20 seconds) following completion of the schedule to prevent overdose. It is important to note, that this time out period has an effect on the behavior of the animal, and makes the protocol not a true continuous reinforcement schedule.

After achieving stable drug delivery, animals are then placed into extinction training sessions, where the drug is no longer available. This period of abstinence allows for the instrumental response to extinguish and a period of abstinence from the drug. This training usually lasts 1-2 weeks and then reinstatement to drug seeking is triggered using cues associated with the drug such as lights and tones (discrete stimuli), contextual stimuli and/or administration of a dose of the drug. Separately, stress can also increase seeking behavior after the response has been extinguished (for a review see (Mantsch, Baker, Funk, Le, & Shaham, 2016)). Interestingly, different ways to prime reinstatement (stress, cue, or drug injection), show little overlap in their

neurobiological mechanisms to induce seeking behavior (Sutton et al., 2003), and are even additive when combined (X. Liu & Weiss, 2002).

Preclinical research uses reinstatement to model drug relapse; however, reinstatement can differ greatly from the clinical population's relapse events. In the clinical population, relapse is considered consumption after a period of abstinence, and the drug is not always consumed in preclinical relapse models. This has led some to point out that these preclinical models are not modeling relapse (Sanchis-Segura & Spanagel, 2006), however they also argue that this doesn't negate their value as a model.

Drug induced alterations of astrocyte biology and morphology.

As referenced above, one of the fundamental cellular consequences of withdrawal from cocaine self-administration is disruption in glutamate homeostasis, mediated by decreased expression and function of GLT-1 and system xC- (Baker et al., 2003; Baker, Shen, & Kalivas, 2002; Kalivas, 2004, 2009; Knackstedt, Melendez, & Kalivas, 2010). Moreover, pharmacological restoration of these systems is associated with decreased reinstatement and behavioral measures of cocaine craving (Knackstedt et al., 2010; Reissner et al., 2014; Reissner et al., 2015; Sepulveda-Orengo et al., 2017). Both of these systems are primarily expressed in astrocytes, suggesting functional significance of cocaine-induced adaptations in astrocytes (Danbolt, 2001; Lehre, Levy, Ottersen, Storm-Mathisen, & Danbolt, 1995; Y. Zhang et al., 2014).

Out of the three types of glial cells (including oligodendrocytes and microglia), astrocytes are the most numerous, outnumbering neurons (Volterra & Meldolesi, 2005) (Allen & Barres, 2009) (Sofroniew & Vinters, 2010)). Astrocytes are star-shaped cells that have multiple functions from nourishing and supporting neurons to roles in synaptic development and signaling, and transmitter uptake and release (for a review, see(Sofroniew & Vinters, 2010)). Astrocytes are classified based on morphology and astrocyte-specific biomarkers such as glial-fibrillary acid protein (GFAP), glutamine synthetase (GS), connexin, and the aquaporin receptor (AQP4). The

most common way to identify astrocytes is GFAP expression, however, not all astrocytes express GFAP (Kimelberg, 2004) (Kettenmann & Ransom, 2012) and GFAP expression is brain region specific (Sofroniew, 2009; Sofroniew & Vinters, 2010). In fact, GFAP is a reliable marker for reactive astrocytes and many astrocytes in healthy CNS do not have detectable levels of GFAP.

Despite similarities in their biomarkers, astrocytes remain a diverse cell type. Astrocytes are heterogeneous cells and the two most prominent types are protoplasmic and fibrous. Despite similarities, they are morphologically and biochemically distinct (Miller & Raff, 1984). Protoplasmic astrocytes are characterized by a large number of branching projections and are primarily located in grey matter, whereas fibrous astrocytes have longer, thinner processes with fewer branches and are primarily located in white matter (Miller & Raff, 1984). Both types make connections with blood vessels through astrocytic end feet and exhibit gap junction coupling with neighboring astrocytes (Kettenmann & Ransom, 2012; Peters, Palay, & Webster, 1991). However, fibrous processes interact with nodes of Ranvier in the white matter tissue, whereas the protoplasmic astrocytes ensheath neuronal synapses in grey matter (Khakh & Sofroniew, 2015). The ends of the astrocytic processes tightly hug the synapse, forming what is known as the tri-partite synapse (Araque, Parpura, Sanzgiri, & Haydon, 1999) (Heller & Rusakov, 2015), engaging in neuronal signaling through the uptake and release of transmitters and trophic factors (Allen, 2014; Appel, Kolman, Kazimirsky, Blumberg, & Brodie, 1997).

For many years it was considered that astrocytes only function was to structurally support and provide nourishment for neural tissue, and that astrocyte reactivity was a marker of diseased tissue, a response to a pathology. It was not considered, until more recently, that astrocyte reactivity could be a causal factor behind these pathologies. Further, Pekny and colleagues (2015) have proposed that there are two pathways of astrogliopathology: (i) the commonly known reactive astrogliosis where astrocytes can have neuroprotective effects or anisomorphic/severe scar forming effects, and (ii) the newer category astrocytopathy that

includes the atrophy/degeneration with loss of function and pathological remodeling of astrocytes (classifications proposed in Pekny review (Pekny et al., 2016), fig 2). Atrophic astrocytes are smaller, do not ensheath the synapse as tightly and have reduced expression of GFAP, AQP4 and glutamate transporter 1 (GLT-1) (for a review see (Pekny et al., 2016)). The consequences of these morphological and biochemical changes include an inability to mediate glutamate homeostasis at the synapse, reduced tone of signaling molecules and trophic factors due to volume distribution and reduced production, altered glucose metabolism and reduced network connectivity of astrocytes (Pekny et al., 2016) (Q. Wang, Jie, Liu, Yang, & Gao, 2017). Interestingly, this pattern closely resembles cellular adaptations that are caused by cocaine.

Cocaine-induced adaptations in astrocytes have recently begun to emerge among the cellular mechanisms which mediate cocaine seeking. The functional consequence of drug-induced adaptations to astrocytes remains largely unknown, and what has been investigated focuses on GFAP expression, which, as stated above, may not be a measure of all astrocytes. It has been reported that short (24 hr) withdrawal from acute administration of non-contingent cocaine increased GFAP expression in the nucleus accumbens shell (NAc shell) but not the core, and both regions had no change in GFAP expression at 1 or 3 week(s) of withdrawal (Bowers & Kalivas, 2003). Also, our laboratory has reported that GFAP is downregulated in the NAc core following cocaine self-administration and extinction (Scofield, Li, et al., 2016). These limited experiments begin to give a picture of dynamic GFAP expression following contingent cocaine exposure, where at early withdrawal points GFAP is increased and at prolonged withdrawal time points we see reduced GFAP expression. These are the first studies with insight into morphological and structural changes that could be occurring in nucleus accumbens astrocytes following cocaine exposure and withdrawal. Further investigation is necessary to make any conclusions on drug-induced astrocyte adaptations.

Of importance, there is some evidence that astroglial asthenia (or atrophy) is characteristic of neuropsychiatric disorders such as frontotemporal dementia, stress, major depressive

disorders, schizophrenia and substance use disorders (Brebner et al., 2005; Niciu, Henter, Sanacora, & Zarate, 2014) (Scofield, Li, et al., 2016) (Braun, Antemano, Helmeke, Büchner, & Poeggel, 2009) (Czeh, Simon, Schmelting, Hiemke, & Fuchs, 2006; Rajkowska & Stockmeier, 2013); for a review see (Scofield, Heinsbroek, et al., 2016; A. Verkhratsky, Nedergaard, & Hertz, 2015), indicating this may be a rich area for future study. There are currently a limited number of pharmacological therapies available for opioid, alcohol and nicotine use disorders, however as stated above there are no FDA approved medications for psychostimulant or cannabis use disorders (NIH, Treatment Approaches for Drug Addiction, Revised July 2016). Accordingly, it is of considerable interest to investigate the potential of psychostimulant-induced adaptations to astrocytes, for the potential development of pharmacotherapies aimed at reducing relapse in substance abuse disorders.

Adaptations in the nucleus accumbens during prolonged withdrawal represent cellular mechanisms that contribute to cocaine seeking.

Use of the rodent self-administration and reinstatement model has yielded considerable information regarding the neurocircuitry and cellular mechanisms associated with cocaine relapse. In particular, the nucleus accumbens (NAc) represents a limbic-motor integrator of the reward circuitry, translating motivation into behavioral output (Mogenson, Jones, & Yim, 1980; Roitman, Wheeler, & Carelli, 2005). As such, the NAc is a particularly salient nucleus for investigation of cellular mechanisms of cocaine seeking, where cocaine-induced adaptations guide cocaine-seeking behavior (Kalivas & Volkow, 2011; Stuber, Hopf, Tye, Chen, & Bonci, 2010; Wolf, 2010). Among these, glutamate homeostasis in the NAc core is disrupted following cocaine experience. Glutamate homeostasis is a balance between glutamate levels in the synaptic and extra-synaptic space, and maintenance of glutamate homeostasis is integral to cellular function (Kalivas, Lalumiere, Knackstedt, & Shen, 2009). Importantly, after cocaine experience and withdrawal, basal extracellular glutamate levels are reduced; however, during

subsequent administration of drug or presentation of drug-associated cues in cocaine-withdrawn animals, glutamate release is transiently, significantly increased (Baker et al., 2003; McFarland, Lapish, & Kalivas, 2003). These disruptions in glutamate homeostasis are largely mediated by reduced expression and activity of glutamate uptake and exchange via system xc- and the high affinity family of glutamate transporters, in particular GLT-1 (Baker et al., 2003; Baker et al., 2002; Fischer-Smith, Houston, & Rebec, 2012; Knackstedt et al., 2010; Pierce, Bell, Duffy, & Kalivas, 1996). The consequence of these disruptions is that during a relapse event, clearance of glutamate from the synapse is impaired, leading to increased tone on glutamatergic receptors in the synaptic and extra-synaptic space, in particular α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptors, driving motivation to seek drug (Backstrom & Hyytia, 2007; Ping, Xi, Prasad, Wang, & Kruzich, 2008; Wolf & Tseng). Supporting this, drugs that upregulate xc-/GLT-1 and normalize glutamate homeostasis attenuate cocaine seeking behaviors after self-administration and extinction (Baker et al., 2003; Knackstedt et al., 2010; Reissner et al., 2014; Reissner et al., 2015; Sepulveda-Orengo et al.).

Additionally, synaptic strength and plasticity are disrupted in NAc medium spiny neurons (MSN) following cocaine experience, including long-term potentiation (LTP) and long-term depression (LTD), which are considered cellular indices of learning and memory (Kasanetz et al., 2010; Martin, Chen, Hopf, Bowers, & Bonci, 2006; Moussawi et al., 2009). Interestingly, despite the resistance to induction of adaptive forms of plasticity following cocaine self-administration and extinction, NAc core synapses are chronically potentiated, as measured by AMPA:NMDA ratios, and become further potentiated after re-exposure to drug-associated cues (Gipson et al., 2013) or the drug itself (Shen, Gipson, Huits, & Kalivas, 2014). Also, inhibition of prelimbic cortical afferents to the NAc attenuates this cue-induced transient potentiation and attenuates cocaine seeking (Stefanik, Kupchik, & Kalivas, 2016). A possible mechanism for this potentiation is increased surface expression of AMPA receptors in the NAc core and shell (Conrad et al., 2008). AMPA receptors in the NAc are integral for cocaine seeking (Briand,

Kimmey, Ortinski, Huganir, & Pierce, 2014; Cornish, Duffy, & Kalivas, 1999; S. L. White et al., 2016) and either pharmacological blockade (Backstrom & Hyytia, 2007; Cornish & Kalivas, 2000; Xie et al., 2012) or reduced surface protein expression (Famous et al., 2008; Ping et al., 2008) attenuates reinstatement to cocaine seeking. However, an intriguing mechanism of AMPA receptor removal that has not been studied in cocaine reinstatement is NMDA receptor triggered endocytosis of AMPA receptors (Biou, Bhattacharyya, & Malenka, 2008; T. C. Brown, Tran, Backos, & Esteban, 2005; Casimiro et al., 2011; Luscher et al., 1999).

Astroglial modulators restore cocaine-induced alterations of synaptic strength and plasticity and influence behavior.

Astroglial modulators that upregulate glutamate transporters GLT-1 and xc- have demonstrated potential as addiction pharmacotherapeutics to restore synaptic strength and plasticity (Reissner et al., 2014; Reissner et al., 2015, Sepulveda-Orengo 2017; Scofield, Li, et al., 2016; Trantham-Davidson, LaLumiere, Reissner, Kalivas, & Knackstedt, 2012). Ceftriaxone (Knackstedt et al., 2010; Trantham-Davidson et al., 2012), N-Acetyl-cysteine (Knackstedt et al., 2009), Propentofylline (Reissner et al., 2015) and Riluzole (Carbone, Duty, & Rattray, 2012) upregulate GLT-1, which is responsible for clearing approximately 90% of all synaptically released glutamate (Kalivas et al., 2009; Tanaka, Ichikawa, Watanabe, Tanaka, & Inoue, 1997), and attenuate cocaine seeking. *N*-Acetyl-cysteine (NAC) increases both GLT-1 and xc- and attenuates cocaine seeking (Baker et al., 2003; Knackstedt et al., 2010), yet this effect is dependent only on restored GLT-1 expression (Reissner et al., 2015). Likewise, propentofylline (Reissner et al., 2014) and ceftriaxone (Knackstedt & Kalivas, 2009; LaCrosse et al., 2017; Trantham-Davidson et al., 2012)attenuate cocaine seeking by upregulation of GLT-1, however the effect of ceftriaxone is also dependent on restored xc- (LaCrosse et al., 2017).

Extending on these studies, we have shown that astrocytes exist in a retracted state and make fewer synaptic connections following two weeks of extinction from cocaine self-

administration, and that the glial modulator ceftriaxone rectifies this deficit (Scofield, Heinsbroek, et al., 2016). These findings expand the appreciation of astrocytes as a component of the model of cocaine-induced deficits in synaptic signaling and plasticity in the NAc and raises the potential of astrocytes as a pharmacotherapeutic target.

Adding to these results, astrocyte derived amino-acid D-serine has been well established as a modulator of learning and memory. D-serine is an important co-agonist of NMDA receptors, which can rescue impairments in learning and memory (H. Han, Peng, & Dong, 2015; Labrie, Wang, Barger, Baker, & Roder, 2010), and facilitate extinction of fear memories (Bai, Zhou, Wu, & Dong, 2014; Labrie et al., 2010; Matsuda et al., 2010), cocaine conditioned place preference (Z. Q. Liu et al., 2016) and cocaine self-administration (Hafenbreidel, Rafa Todd, Twining, Tuscher, & Mueller, 2014; Kelamangalath, Seymour, & Wagner, 2009; Kelamangalath & Wagner, 2010).

We have recently shown that astrocytes retract from synapses in the NAc core following cocaine self-administration and withdrawal (Scofield, Li, et al., 2016), creating a potential barrier for volume transmission of D-serine to the synapse (Panatier et al., 2006). A reduction of D-serine at the synapse may thus impair NMDA receptor function, contributing to the increase in AMPA:NMDA ratio observed in the NAc of cocaine-withdrawn animals (Gipson et al., 2013; Shen et al., 2014). Importantly, a change of NMDA receptor function due to decreased D-serine tone would be independent of NMDA receptor subunit expression. In fact, some studies have shown upregulated NMDA receptor expression following cocaine self-administration and extinction (Hafenbreidel et al., 2014; P. I. Ortinski, 2014) and forced abstinence (Lu, Grimm, Shaham, & Hope, 2003; Tang, Wesley, Freeman, Liang, & Hemby, 2004). This cocaine-induced, astrocyte-mediated reorganization of synaptic function is at the heart of my dissertation project.

Goals of the current dissertation.

The finding that NAc core astrocytes are smaller and make fewer synaptic contacts in cocaine- versus saline-extinguished animals raises interesting and important questions regarding the functional significance of this drug-induced adaptation. The governing hypothesis that was tested in this dissertation project was that reduced synaptic colocalization of astrocyte processes creates a barrier to volume transmission of astrocyte-derived D-serine to neuronal NMDA receptors, thereby contributing to the synaptic adaptations which characterize cocaine seeking behaviors. Consequently, the primary goal of this dissertation was to characterize the effect of D-serine augmentation on reinstatement to cocaine seeking. To accomplish this, two strategies were utilized: (i) characterization of the behavioral effects of D-serine and (ii) elucidation of the cellular and molecular mechanisms responsible for the observed effects. Chapter 2 provides an assessment of the behavioral effect of D-serine augmentation on cocaine seeking. Chapter 3 investigated the role of the NAc in the behavioral effects of systemic D-serine augmentation, using NAc-specific stimulation of NMDA receptors, followed by cell surface biotinylation to assess the effects of systemic D-serine augmentation on cell-surface glutamate receptor expression in the NAc. Finally, in Chapter 3, I determined the effects of D-serine augmentation on synaptic strength and plasticity in the NAc utilizing whole cell patch clamp electrophysiology.

Importantly, I hypothesized that D-serine augmentation, during a cocaine plus cue-primed reinstatement test, will increase tone on NMDA receptors. I hypothesized that the combination of D-serine with elevated levels of glutamate released during reinstatement would induce NMDA receptor-mediated AMPA endocytosis, depotentiate the synapse and reduce the cellular excitation of the NAc that drives reward seeking. Thus, the impetus of this dissertation was to investigate the cellular mechanism by which astrocyte-derived gliotransmitter D-serine can reduce cocaine reward and seeking. Results obtained collectively from these studies add to the discussion of whether NMDA receptor agonism can impair cocaine seeking and normalize

synaptic strength in the NAc. Importantly, the results generated by the experiments in this dissertation will inform future hypotheses and guide understanding of how astrocytes modulate neuronal processing and synaptic plasticity, as well as elucidate the potential mechanism for these changes.

CHAPTER 2:

The Effects of D-serine Augmentation on Cocaine Seeking.

Introduction.

Relapse to drug seeking after a period of abstinence is a defining feature of substance use disorders. Preclinical animal studies indicate that withdrawal from chronic cocaine use is characterized by cellular adaptations and structural remodeling within the brain's reward circuitry, which are believed to drive persistent drug seeking and relapse. Accordingly, identification of cellular adaptations which mediate drug seeking represent candidate targets for pharmacotherapeutic development.

As introduced in the preceding chapter, chronic cocaine self-administration and withdrawal lead to maladaptive adaptations within the NAc core. The NAc core is an important limbic-motor integrator that translates internal motivational states into motor output (Mogenson et al., 1980; Roitman et al., 2005), and as such is central to relapse behaviors (McFarland and Kalivas 2001). Among these functionally significant adaptations, withdrawal from cocaine self-administration is characterized by impaired glutamate homeostasis in the NAc core, and interventions that rectify this dysregulation inhibit cocaine seeking [for a review see (Scofield & Kalivas, 2014)]. Because glutamate homeostasis is largely mediated by the transporter and anti-porter systems localized on astrocytes, a greater appreciation of astrocyte-mediated mechanisms of cocaine seeking is warranted.

Astrocytes make close physical contact with neuronal synapses, referred to as the tripartite synapse (Araque et al., 1999) and have been extensively implicated in synaptic transmission, strength and plasticity (De Pitta et al.; Haydon & Nedergaard, 2015; Panatier et al.,

2006). In particular, NMDA receptor activity is gated by co-agonism by D-serine in the nucleus accumbens (Curcio et al., 2013).

D-serine is one in a list of gliotransmitters that are released by astrocytes including GABA, adenosine, and others (Araque et al., 1999; Barker & Ullian, 2010). The biosynthetic enzyme serine racemase (SR) converts L-serine to D-serine and is found both in neurons and astrocytes (Curcio et al., 2013; Verrall et al., 2007; Wolosker, 2011), providing evidence that D-serine is not exclusively a gliotransmitter. However, the first step of L-serine production is catalyzed by 3-phosphogylcerate dehydrogenase (3-PGDH), an exclusively astrocytic enzyme (Ehmsen et al., 2013; Martineau, Parpura, & Mothet, 2014). The serine shuttle model proposes that L-serine is generated in astrocytes and shuttled to neurons using alanine-serine-cysteine-1 transporter-1 (Asc1) and alanine/serine/cysteine/threonine transporter (ASCT; (Wolosker, 2011)) to be synthesized by SR. Importantly, D-serine is the primary endogenous co-agonist of the N-methyl-D-aspartate (NMDA) receptor at the glycine modulatory site in the NAc, as the effect of glycine degradation on NMDA-mediated EPSCs is negligible in this region (Curcio et al., 2013).

It is important to note that the role of astrocytes in D-serine synthesis and transmission is controversial. Recent evidence has pointed to neurons, not astrocytes, as the primary source of SR, the bidirectional enzyme that converts L-serine to D-serine and vice versa (Foltyn et al., 2005), and SR knockout studies indicated a reduction in D-serine in neurons and not astrocytes (Benneyworth & Coyle, 2012). Additionally, there has yet to be evidence supporting D-serine release machinery in astrocytes [(Agulhon, Fiacco, & McCarthy, 2010) for a review see (Wolosker, Balu, & Coyle, 2016)]. Because of this, Wolosker and colleagues support neurons as the dominant source of D-serine (Wolosker et al., 2016). Countering these claims, Oliet and colleagues point to established research that indicates astrocytes have optimal conditions for D-serine synthesis, and neurons have optimal conditions for D-serine degradation (Ehmsen et al., 2013; Foltyn et al., 2005; Papouin, Henneberger, Rusakov, & Oliet, 2017). They further argue

that differences in experimental conditions from physiological conditions can reverse serine racemase to primarily degrading D-serine and that using staining methods for serine racemase in neurons and astrocytes is confounded by the difference in makeup of the neruopile [~5-5% astrocytic and 70-75% neuronal; (Papouin et al., 2017)]. Finally, they point out that despite a lack of evidence for release machinery in astrocytes, there is also currently no evidence for release mechanisms of D-serine in neurons. As such, further investigation is necessary to settle this dispute of the locus, either astrocytic or neuronal, of D-serine synthesis and release.

Our lab has recently reported that NAc core astrocytes exist in a retracted state following extinction from cocaine self-administration, where the astrocytes are reduced in surface area, volume and co-localization to the synapse compared to saline control animals (Scofield, Li, et al., 2016). These findings follow a growing amount of research implicating atrophic astrocytes in neuropsychiatric disorders such as major depressive disorder, schizophrenia and chronic stress (Katsel et al., 2011; Rajkowska & Stockmeier, 2013; Q. Wang et al., 2017; Y. Zhao et al., 2017). Interestingly, administration of ceftriaxone, a beta-lactam antibiotic, known to attenuate cocaine reinstatement by rectifying glutamate homeostasis (Kalivas, 2009; Knackstedt et al., 2009), reverses the deficit in synaptic co-localization of NAc core astrocyte peripheral processes in cocaine self-administering animals (Scofield, Li, et al., 2016). As such, combating these enduring cellular adaptations caused by cocaine use and prolonged withdrawal, such as atrophic astrocytes, could be useful targets to reduce relapse.

One of the consequences of atrophic astrocytes is impaired functional release of gliotransmitters. Identified astrocyte-derived factors include glutamate, GABA, ATP, D-serine, and others (Alexei Verkhratsky, Matteoli, Parpura, Mothet, & Zorec, 2016). Subsequently, D-serine can be transported to neurons, and can be released by either neurons or astrocytes. (Martineau et al., 2014; Papouin et al., 2017; Wolosker et al., 2016). Reduced synaptic colocalization of astrocyte processes results in a barrier of volume transmission for both L- and D-serine, raising the hypothesis that NMDA function mediated by D-serine co-agonism is

impaired in cocaine-extinguished animals, and contributes to the cellular adaptations which drive maladaptive synaptic and behavioral function.

Supporting this, D-serine administration reduces behavioral sensitization to non-contingent cocaine (Curcio et al., 2013; Z. Q. Liu et al., 2016; Puhl, Berg, Bechtholt, & Coyle, 2015). Additionally, D-serine reduces compulsive alcohol intake (Seif et al., 2015). Given the important role for D-serine in NMDA receptor function and learning and memory, D-serine has also been shown to facilitate extinction of fear memories (Bai et al., 2014), morphine and cocaine conditioned place preference (Dias, Wang, & Phillips, 2012; Z. Q. Liu et al., 2016; Puhl et al., 2015), and cocaine self-administration (Hafenbreidel et al., 2014; Kelamangalath et al., 2009; Kelamangalath & Wagner, 2010). Despite these advances, D-serine has yet to be studied as a pharmacotherapy for relapse after protracted withdrawal. Therefore, the first goal of this study was to determine if D-serine augmentation would impair reinstatement to cocaine seeking. In order to augment D-serine co-administration of D-serine and sodium benzoate (SB), an inhibitor of the metabolizing enzyme of D-Serine [D-Amino Acid Oxidase (DAO)] were given systemically prior to a test of reinstatement to reward seeking.

Methods.

Animals.

Male Sprague-Dawley rats (Harlan Farms, Raleigh NC), aged approximately 6-8 weeks and weighing 260-300 grams at the time of surgery were used in these experiments. Rats were individually housed in a temperature controlled environment on a reversed 12:12 light:dark (lights off at 0700) schedule. Following a week of habituation, animals were put on a food restricted diet of 20g/day (Envigo Teklad laboratory animal diet). All procedures were approved by the University of North Carolina's Institutional Animal Care and Use Committee. One animal was removed from the food reinstatement analyses because the animal had spontaneous recovery of food seeking behavior the day before reinstatement test.

Catheterization surgery.

Animals were anesthetized with ketamine (100 mg/kg) and xylazine (7mg/kg) and given ketorolac analgesic (0.28-0.32 mg/kg) for catheterization surgery. Briefly, chronic indwelling catheters were constructed from 23-cm Bio-sil Silastic catheter (0.02mm inner diameter, 0.047mm out diameter; Dow Corning, Midland, MI) with a 22 gauge cannula (Plastics One, Wallingford, CT). Catheters were implanted into the right jugular vein for the administration of intravenous (*iv*) cocaine and exited the animals' back between the scapulae, as described previously (Scofield, Heinsbroek, et al., 2016). Animals received 5 days of recovery and catheters were flushed daily with antibiotic (gentimicin 5mg/ml, 0.1 ml, *iv*) followed by heparinized saline (100 mg/ml, 0.1 ml, *iv*) during the recovery period and throughout self-administration training. Catheters were checked for patency with propofol (10mg/kg, SAGENT Pharmaceuticals, Schaumburg, IL).

Behavioral training.

Self-Administration and Extinction Training.

Self-administration training was conducted in standard operant conditioning chambers (Med Associates Inc.) that contained a white house light, two retractable levers, and a bar floor. Rats were first trained to lever press for food in a food training session where criteria were set at 6 hr or delivery of 100 pellets. After recovery from surgery, animals were then trained to lever press for cocaine infusions (cocaine hydrochloride; 0.2 mg per infusion, *iv*; NIDA) under a fixed ratio (FR1) reinforcement schedule with a discrete stimulus complex during infusion and 20*s* time out. The stimulus complex consisted of a tone (70 dB, 2.5 kHz) and a stimulus light above the active lever activated for 5 *s* during the infusion. There was a second lever that had no programmed response when pressed; this served as a control for lever responding throughout the experiment. Rats were trained to self-administer cocaine in 2 hr daily sessions for 12 days or until they

achieve the self-administration criterion (10 days of greater than 10 infusions per session). A subset of animals were trained to self-administer food (Purina Precision Rodent Chow, 45 mg/pellet) or sucrose (Purina Precision Sucrose, 45 mg/pellet) pellets on an escalating FR1:FR3:FR5 and complex cue schedule used previously to test for natural reward seeking (Cosme, Gutman, & LaLumiere, 2015; McFarland et al., 2003). Rats in these experiments did not receive a food training session prior to self-administration training and during self-administration training their reinforcers were paired with the same complex cues as the cocaine experiments. Treatment groups were balanced based on the number of active lever presses during the last 3 days of self-administration. Following self-administration, animals either received extinction training or forced abstinence where animals remained in their home cage for 2 weeks and were handled at least 3 times a week. During extinction training, responding on either lever had no programmed result. Rats received 14 extinction training sessions before the test of reinstatement.

Tests of reinstatement or extinction.

Cocaine- Plus Cue-primed Reinstatement or Extinction Test

Twenty-four hr after the last extinction training session rats were tested for reinstatement of cocaine-seeking behaviors in a 2 hr test session. In a separate experiment, rats who did not receive extinction training, but remained in the home cage for two weeks, were tested for cocaine-seeking behaviors in a 2 hr test session at the same time as their self-administration sessions (extinction test. In both cases, rats received a cocaine prime (10 mg/kg, *i.p.*) immediately prior to the session. During the test session, lever responses were recorded without cocaine reinforcement. The stimulus complex was presented identical to self-administration training on lever press of the active lever on an FR1 schedule. Inactive lever responses were recorded but had no programmed response. A subset of animals in the acute

treatment experiment received cue alone or cocaine alone reinstatement tests (data not shown) before the cocaine plus-cue primed reinstatement test.

Food or Sucrose Plus Cue-primed Reinstatement Test

To emulate the cocaine and cue-primed reinstatement test, rats who received food or sucrose self-administration training received a food or sucrose plus cue-primed test of reinstatement to reward seeking, as described previously (Cosme et al., 2015). At the start of the session 2 pellets were placed in the food hopper and then every 2 minutes one pellet was delivered non-contingently for 30 minutes. Throughout the 2-hr test the complex cue previously associated with food or sucrose reinforcement was available on an FR1 schedule similar to the tests of cue-primed tests of cocaine seeking.

Locomotor test.

24 hr following reinstatement test, a subset of rats were tested for locomotor activity of habituation to a novel environment and a cocaine challenge. Standard locomotor chambers (Med Associates Inc.) with beams to track locomotor activity were used. Locomotor testing was conducted 3 hr after treatment of D-serine augmentation or vehicle. Rats were placed in the center of the chamber and the 2 hr locomotor test began. Sixty minutes into the locomotor test the session was paused for a cocaine injection (10 mg/kg, *intraperitoneal, i.p*). Locomotor activity was collected in 5 minute bins in mm of ambulatory activity.

Treatment.

Rats received either acute or 3-day D-serine augmentation treatment of D-serine and sodium benzoate (DAO inhibitor). 3-Day D-serine augmentation was administered systemically 3 hr prior to the last two extinction sessions (100 mg/kg D-serine*, i.p.*; 100 mg/kg SB, *i.p.*) and on reinstatement or extinction test day (100 mg/kg D-serine, *i.p.*; 200 mg/kg SB, *i.p.*). Acute D-

serine augmentation was administered 3 hr before the reinstatement test session (100 mg/kg D-serine, *i.p.*; 200 mg/kg SB, *i.p.*).

D-serine measurements.

To determine the effect of systemic D-serine augmentation on D-serine levels in the NAc, a subset of animals received behavioral training as described, but no test of reinstatement. Rats were sacrificed by rapid decapitation 3 hrs after the third treatment (when reinstatement testing would begin) and the NAc core was dissected with a 2 mm punch and flash frozen using dry ice and isopentane. Neutralized perchloric acid extract and high-pressure liquid chromatography (HPLC) measurements were performed by The CHOP Metabolomics Core directed by Dr. Itzhak Nissim (https://metabolomic.research.chop.edu). Mass spectrometry was then used to determine measurements of D-serine, kynurenic acid, and kynenurine. Analysis was performed as previously described (Nissim et al., 2014; Wimmer et al., 2017).

Data analysis.

A 2x2 repeated measures analysis of variance (ANOVA) was used to compare the behavioral data of the groups (active lever responding, inactive lever responding and infusions) with Sidak's multiple comparison test *post hoc* corrections to compare groups. Student's unpaired two-tailed t-test was used to compare extinction test data of two groups. HPLC analysis used a one-way ANOVA with Sidak's multiple comparison test *post hoc* corrections to compare groups. Alpha was set at $p=0.05$ for all statistics.

Results.

3-Day systemic D-serine augmentation attenuates cocaine and cue-primed test of reinstatement.

In order to determine the effect of D-serine augmentation on cocaine seeking, animals were trained in cocaine self-administration followed by extinction and a cocaine plus cue-primed reinstatement test. During self-administration no differences were observed between animal groups (Figure 2.1B) in lever responding ($F_{(5.49)}$=0.4, p=0.86, active; $F_{(5.49)}$=0.9, p=0.50, inactive) and cocaine infusions ($F_{(5.49)}$=0.4, p=0.856), however there was a main effect of session on cocaine infusions ($F_{(9.450)}$=15, p<0.01). There was no difference in lever responding during extinction training between the groups ($F_{(5.49)}$=0.3, p=0.90) and all groups extinguished active lever responding during extinction training (Sidak's multiple comparison test, p<0.05; Figure 2.1B).

All groups significantly reinstated cocaine seeking during a cocaine plus cue-primed test compared to the last day of extinction training (interaction effect (IE) $F_{(5,49)}$=5.5, p<0.02; main effect (ME) of session $F_{(1,49)}$=138.3, p<0.01, Figure 2.1B). There was a main effect of treatment ($F_{(5,49)}$=3.6, p<0.01) and *post hoc* comparisons revealed that rats who received systemic D-serine augmentation reinstated cocaine seeking significantly less than rats treated with vehicle or SB (200mg/kg) alone (p<0.01, Figure 2.1C).

Acute Systemic D-serine augmentation has no effect on reinstatement to cocaine seeking.

In this experiment, rather that received systemic administration of D-serine and SB prior to the last two extinction sessions as well as reinstatement test, a single administration was give immediately prior to the reinstatement test only. Self-administration training behavior was the same between all treatments (Figure 2.2A), with no differences in lever responding ($F_{(2.19)}$=0.7, p=0.50, active; $F_{(2,19)}$=0.8, p=0.47, inactive) or cocaine infusions ($F_{(2.18)}$=1.5, p=0.26). Additionally, there was no difference between treatment groups in extinction training ($F_{(2,19)}$=0.8,

p=0.08, active; $F_{(2,19)}$=0.1, p=0.89, inactive) and all groups extinguished active lever responding during extinction training (Sidak's multiple comparison test, p<0.05; Figure 2.2B).

All groups significantly reinstated cocaine seeking during a cue plus cocaine primed test compared to the last day of extinction training (IE $F_{(2,18)}$=0.6, p=0.58, ME of session $F_{(1,18)}$=70.9, *p*<0.01) and there was no difference in cocaine seeking between the groups on test day ($F_{(2,18)}$=0.6, p=0.53, Figure 2.2C).

Following acute treatment of the low dose of D-serine augmentation (100 mg/kg D-serine + 100 mg/kg SB) animals were tested for locomotor activity. As expected, both groups habituated to the novel environment in the first 60 minutes of the activity test (ME of time $F_{(23.322)}$=69.9, *p*<0.01). Surprisingly, there were significant differences between the treatment groups in their locomotor activity (IE $F_{(23,322)}$=1.6, *p*<0.05; ME of treatment $F_{(1.14)}$=11.2, *p*<0.01). After a cocaine challenge at 60 minutes, post hoc analyses indicate that the acute D-serine augmentation group had significantly lower locomotor activity compared to vehicle animals (D-serine augmentation > Vehicle minutes 65-75, and 200; Sidak's multiple comparisons test, *p*<0.05, data not shown). Although unexpected, given the null effect of acute treatment on cocaine seeking behavior, these results are in parallel with previously published work that acute doses of D-serine attenuate locomotor sensitization in a model of non-contingent cocaine exposure (Curcio et al., 2013).

D-serine augmentation increases D-serine levels compared to control animals after 3-days of treatment but not acute treatment.

I hypothesize that the effect of systemic D-serine augmentation on cocaine reinstatement is mediated by cellular effects in the NAc core. As a first step toward testing this hypothesis, I first measured the effect of systemic D-serine augmentation on D-serine levels in the NAc core in cocaine-extinguished animals.

Behavioral training (Figure 2.3B) was the same among treatments for self-administration of cocaine ($F_{(2,21)}$=0.9, p=0.43, active lever responding; $F_{(2,21)}$=1.2, p=0.33, inactive lever responding; $F_{(2,21)}$=0.4, p=0.65 cocaine infusions and extinction training ($F_{(2,21)}$=0.4, p=0.68, active lever responding; $F_{(2,21)}$=1.2, p=0.33, inactive lever responding). All groups extinguished active lever responding during extinction training (ME of session $F_{(13,273)}$=26.1, p<0.01). Rats that received 3 days of D-serine augmentation had significantly higher D-serine levels than control animals, where treatment with only 1 day of D-serine augmentation was not significantly different than vehicle ($F_{(2,21)}$=4.4 p<0.03*;* Sidak's multiple comparisons test p<0.02; Figure 2.3C*)*.

Because inhibition of D-amino acid oxidase can also affect levels of kynurenic acid (Ayala 2015), an inhibitor of NMDA receptors, we measured kynurenic acid and its precursor kynurenine in the same group of animals. Kynurenic acid has been linked to cocaine addiction research (Badawy, 2017; Vengeliene, Cannella, Takahashi, & Spanagel, 2016; Witkin, 1993). There were no differences in levels of kynurenic acid or kynurenine between vehicle and 3-day or acute treatment with D-serine augmentation ($F_{(2,21)}$=0.1, p=0.90 kynurenic acid; $F_{(2,21)}$=1.8 p=0.20, kynurenine; Figure 2.3D-E).

3-Day Systemic D-serine augmentation reduces cocaine seeking in a model of forced abstinence, but not locomotor activity.

D-serine augmentation has been found to enhance extinction learning and attenuate reinstatement levels (Hammond, Seymour, Burger, & Wagner, 2013; Kelamangalath et al., 2009; Kelamangalath & Wagner, 2010). To confirm that the treatment given here was effecting reinstatement mechanisms and extinction learning, rats received 3-day D-serine augmentation in a model of forced abstinence, in which rats did not receive extinction training.

No differences were found in self-administration training between treatment groups in cocaine self-administering ($F_{(1.24)}$=0.2, p=0.68, cocaine active lever responding; $F_{(1,24)}$=1.2, p=0.28, cocaine inactive lever responding; $F_{(1,24)}$=0.3, p=0.59 cocaine infusions; Figure 2.4B),

Following self-administration and forced abstinence, D-serine augmented animals exhibited significantly attenuated cocaine seeking during a cocaine plus cue-primed test of extinction compared to control animals ($T_{(24)}$=2.4, p<0.03; Figure 2.4D). These results indicate that the effect of D-serine augmentation on cocaine seeking is independent of extinction training, and thus the previous results were unlikely to be a consequence of facilitated extinction learning.

To determine if the effect of 3 days of D-serine augmentation on reinstatement is a true effect of seeking behavior and not an artifact of reduced activity, a subset of rats was given a locomotor activity test. Both treatment groups (D-serine augmentation and vehicle) habituated to the novel environment and showed increased activity following cocaine challenge (IE $F_{(23,322)}$=2.5, p=0.18; ME of Time $F_{(23,161)}$=21.6, p<0.01), but treatment had no effect on locomotor activity ($F_{(1,7)}$=0.8, p=0.4, Figure 2.4E).

D-serine augmentation has no effect on sucrose seeking or food seeking.

Additionally, to determine whether the treatment affects all rewards we tested 3-day D-serine augmentation's ability to affect sucrose and food seeking in a model of self-administration and extinction training. Sucrose and food self-administering animals had no differences in self-administration training behavior ($F_{(1.13)}$=0.1, p=0.71, sucrose active lever responding; $F_{(1,13)}$=0.113, p=0.7421, sucrose inactive lever responding; $F_{(1.13)}$=0.0021, p=0.9637 sucrose reinforcers, $F_{(1.14)}$=0.1228, p=0. 7313, food active lever responding; $F_{(1,14)}$=0.01654, p=0.8995, food inactive lever responding; $F_{(2.18)}$=0.0001136, p=0.9916 food reinforcers; Figure 2.5B&D) or extinction training ($F_{(1.14)}$=2.157, p=0.1641, sucrose active lever responding; $F_{(1,14)}$=0.01788, p=0.8955, sucrose inactive lever responding; $F_{(1,14)}$=1.789, p=0.2024, food active lever responding; $F_{(2,18)}$=0.102, p=0.7542, food inactive lever responding) and all groups extinguished seeking behavior (Tukey's multiple comparisons test, p<0.05).

D-serine augmentation had no effect on reinstatement to other rewards. There were no differences in food seeking between the treatment groups during a food plus cue-primed test of

reinstatement (IE $F_{(1,13)}$=1.3, p=0.27; ME of Session $F_{(1,13)}$=66.08, p<0.0001, Treatment $F_{(1,13)}$=0.2247, p=0.6434; Figure 2.5C). Additionally, sucrose seeking was not affected by treatment with D-serine augmentation during a sucrose plus cue-primed test (IE $F_{(1,13)}$=3.0, p=0.11; ME of session $F_{(1,13)}$=32.33, p<0.0001, ME of treatment $F_{(1,13)}$=2.622, p=0.1294; Figure 2.5E). These results are strong indicators that D-serine augmentation does not affect motivation to seek all rewards.

Discussion

Systemic D-serine augmentation impairs cocaine seeking

D-serine has been shown to oppose cocaine locomotor sensitization (Curcio et al., 2013; Z. Q. Liu et al., 2016), attenuate cocaine conditioned place preference (Hammond et al., 2013; Z. Q. Liu et al., 2016) and attenuate reinstatement to cocaine seeking through facilitation of extinction memories (Curcio et al., 2013; Hammond et al., 2013; Kelamangalath et al., 2009; Kelamangalath & Wagner, 2010). The current findings expand on the role of D-serine administration to attenuate relapse-related behaviors by acting on mechanisms of relapse directly. While 3 days of augmentation of D-serine attenuated cocaine seeking, via D-serine administration together with an inhibitor of D-amino acid oxidase, acute augmentation of D-serine did not affect reinstatement. Moreover, I found that 3 days of D-serine augmentation was required to significantly raise levels of D-serine in the NAc core, an important brain region in relapse to reward seeking (McFarland & Kalivas, 2001), whereas a single administration did not affect NAc D-serine levels. Additionally, the effects of 3-day D-serine augmentation on cocaine seeking were not a result of impaired locomotor behavior (Figure 2.1D). Locomotor activity was unchanged when habituating to a novel environment and following a cocaine challenge dose.

Previous studies have shown that D-serine administration can enhance extinction to cocaine seeking (Hafenbreidel et al., 2014; Kelamangalath et al., 2009). These studies differ from our own, in that D-serine was administered together with all extinction sessions. In our

studies, lever responding was extinguished well before administration of the treatments, and therefore the mechanism of action is likely independent of any learning during the advanced extinction training sessions. Thus, while previous studies have found that D-serine augmentation can facilitate extinction learning, extinction was already thoroughly consolidated before administration was performed.

In order to more fully dissociate the role of extinction in the effects of D-serine on attenuation of cocaine seeking, D-serine treatment was tested in a model of forced abstinence, instead of extinction. D-serine augmentation similarly attenuated reinstatement to cocaine seeking as in animals that received extinction training, indicating that D-serine augmentation in our model is likely affecting mechanisms of reinstatement. Supporting this, administration of D-serine alone during extinction training in a novel context had no effect on reinstatement behavior (Hammond et al., 2013) and administration during only early extinction, but not chronically throughout extinction training, had no effect on a test of cocaine seeking (Hafenbreidel et al., 2014), adding insight into our results that we are directly targeting reinstatement mechanisms.

The effects of D-serine and sodium benzoate on NAc D-serine and kynurenic acid levels

D-serine augmentation combined D-serine with a drug that inhibits the enzyme that breaks down D-serine and catalyzes kynurenic acid (NMDA receptor antagonist) to directly manipulated the mechanisms of reinstatement through elevated D-serine levels during the reinstatement test. We predict that, with our combination of drugs, D-serine was elevated to a greater extent than administration of D-serine alone, and D-serine continued to be elevated for a greater length of time, thus having a significant impact on NMDA receptor activation. In support of this, previous experiments show that following systemic administration brain levels of D-serine are increased 24 hrs later and return to basal levels in 3 days (Hashimoto & Chiba, 2004), and thus administration on consecutive days would have an additive effect on brain D-serine levels.

Kynurenic acid is a primarily astrocyte-derived (Guillemin et al., 2001) potent inhibitor of NMDA receptors (Alkondon et al., 2011; Kessler, Terramani, Lynch, & Baudry, 1989; Moroni, 1999);. As discussed, DAO also catalyzes kynurenic acid, and so it is possible that our administration of D-serine and SB could both augment D-serine and inhibit kynurenic acid levels. However, there were no differences in kynurenic acid levels in the NAc core compared between saline- and cocaine-extinguished rats. The null effects could be a result of insufficient sensitivity of our measure due to the low concentration. Additionally, it is important to note that DAO is just one enzyme that catalyzes kynurenine into kynurenic acid, for instance there is a family of kynurenine aminotransferases that catalyze kynurenic acid (Q. Han, Cai, Tagle, & Li, 2010). Thus, it is possible that only inhibiting DAO was insufficient to affect the levels of these molecules. Further, we systemically injected the DAO inhibitor SB, and although we found no difference in levels in the NAc core, this may not be the case in other regions where kynurenic acid has plentiful targets, such as the prefrontal cortex.

D-serine augmentation is without effect on food or sucrose reinstatement

D-serine augmentation has no effect on reinstatement to non-drug reward seeking behaviors, although, there was a slight trend for sucrose seeking to be decreased (Figure 2.5E; p=0.13). While sucrose has traditionally been considered a more natural reward in comparison to cocaine, accumulating evidence indicates that sucrose reward can induce cellular adaptations similar to cocaine. For example, repeated sucrose ingestion leads to potentiation of NAc core synapses through up regulation of GluA1 containing AMPA receptors (Oginsky, Goforth, Nobile, Lopez-Santiago, & Ferrario, 2016; Tukey et al., 2013), Similarly, NAc core synaptic potentiation has been reported in obesity-prone rats following access to a palatable diet (R. M. Brown et al., 2017; Oginsky et al., 2016). It's noteworthy that following sucrose self-administration and extinction NAc core synapses do not elicit the cue-induced transient potentiation that has been associated with cue-induced cocaine seeking (Gipson et al., 2013)

and further, NAc core PL afferents express increased-fos, a marker of neuronal activity, following cocaine cue-induced reinstatement but not after sucrose cue-induced reinstatement (McGlinchey, James, Mahler, Pantazis, & Aston-Jones, 2016). Accordingly, D-serine augmentation may attenuate sucrose-induced changes at the synapse and hence impair sucrose reinstatement, similar to our proposed mechanism for cocaine seeking, but these hypotheses warrant further investigation. Importantly, in food seeking these synaptic changes are not present (Cosme et al., 2015) and D-serine augmentation has no effect on reinstatement to food seeking, signifying that D-serine augmentation does not affect all rewards.

Influence of reinstatement modality

These experiments utilized a cocaine plus cue-primed test of reinstatement to reward seeking behavior. This combination of the subjective effects of the drug or palatable reward as well as the cues associated with the reward are powerful motivators to relapse to seeking behavior. The mechanisms by which each of these motivators affect reinstatement behavior have been found to be additive, and therefore possibly affecting different mechanisms (Sanchis-Segura & Spanagel, 2006). In these experiments, we cannot determine if D-serine augmentation was acting on effects of a cocaine challenge dose, the cocaine conditioned cues, or both during the reinstatement test. Thus, future studies should determine the efficacy of D-serine augmentation to attenuate either prime alone.

Alternatively, it has been found a robust glutamate efflux in the NAc and mPFC during a methamphetamine plus cue reinstatement test (Parsegian & See, 2014), and it is possible that the release of glutamate during a single prime event is not as robust. Parsegian (2013) did not make direct comparisons of meth plus cue-prime to meth-prime or cue-prime alone, and differences in glutamate release following cocaine or cocaine plus cue prime have not been investigated. However, if there is increased glutamate release when both a drug prime and a cue prime are given simultaneously, this could provide great insight into the robust nature of

drug plus cue-primed reinstatement tests, which tend to have much higher responding than either prime alone. The mechanism by which D-serine augmentation is attenuated seeking behavior may be dependent on robust glutamate release in combination with the added exogenous D-serine to vigorously activate NMDA receptors, thus decreasing seeking behavior.

The locus of the effect of D-serine augmentation and the mechanism by which D-serine augmentation is working remain elusive and future studies need to address these questions. Because the injections were given systemically, it is not possible to determine if the NAc is the brain region driving this effect, or if it is involved in any manner. To determine this, additional studies described below will directly administer D-serine into the NAc to determine if activation of NAc NMDA receptors are sufficient for this effect. Further, these behavioral studies cannot determine the mechanism by which NMDA receptor activation during a reinstatement test is attenuating cocaine seeking behavior. Future studies should investigate this mechanism of action, to aid in the development of pharmacotherapeutic relapse treatments.

Figure 2.1. 3-Day D-serine augmentation attenuates cocaine plus cue-primed reinstatement to cocaine seeking. (A) Timeline of the study. (B)Treatment groups did not differ during self-administration training and extinction. (C) 3 Day treatment with D-serine (DS, 100 mg/kg) and sodium benzoate (SB; 200 mg/kg) significantly attenuated cocaine seeking compared to vehicle treated animals in a cocaine plus cue-primed reinstatement test. Animals treated with either a lower dose of SB in combo with DS (100 mg/kg, both) or either drug alone DS (100 mg/kg) or sodium benzoate (100 mg/kg, 200 mg/kg) were no different in active lever responding compared to vehicle-treated animals. Hash denotes significant effect of reinstatement (#p<0.05); Asterisk denotes significant effect *vs.* vehicle (*p<0.05); $ denotes significant effect *vs.* 200 mg/kg SB ($*p*<0.05).

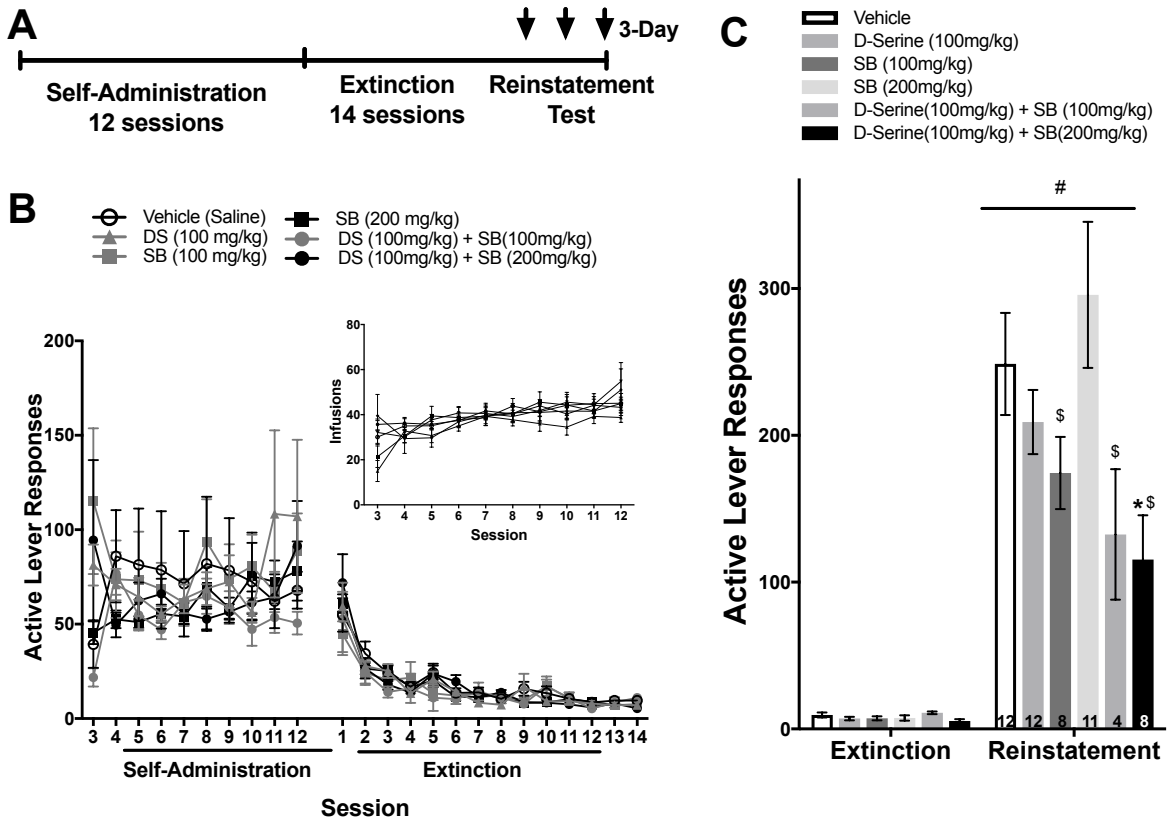


Figure 2.2. Acute D-serine augmentation has no effect on reinstatement to cocaine seeking. (A) Study timeline, some animals also received additional reinstatement tests of either cocaine alone or cue alone (data not shown). (B) There were no differences between treatment groups in self-administration or extinction training. (C) Acute administration of D-serine augmentation (100 mg/kg DS + 200 mg/kg SB) or (100 mg/kg DS + 100 mg/kg SB) had no effect on cocaine seeking during a cocaine plus cue-primed reinstatement test. Hash denotes significant effect of reinstatement (#p<0.05).

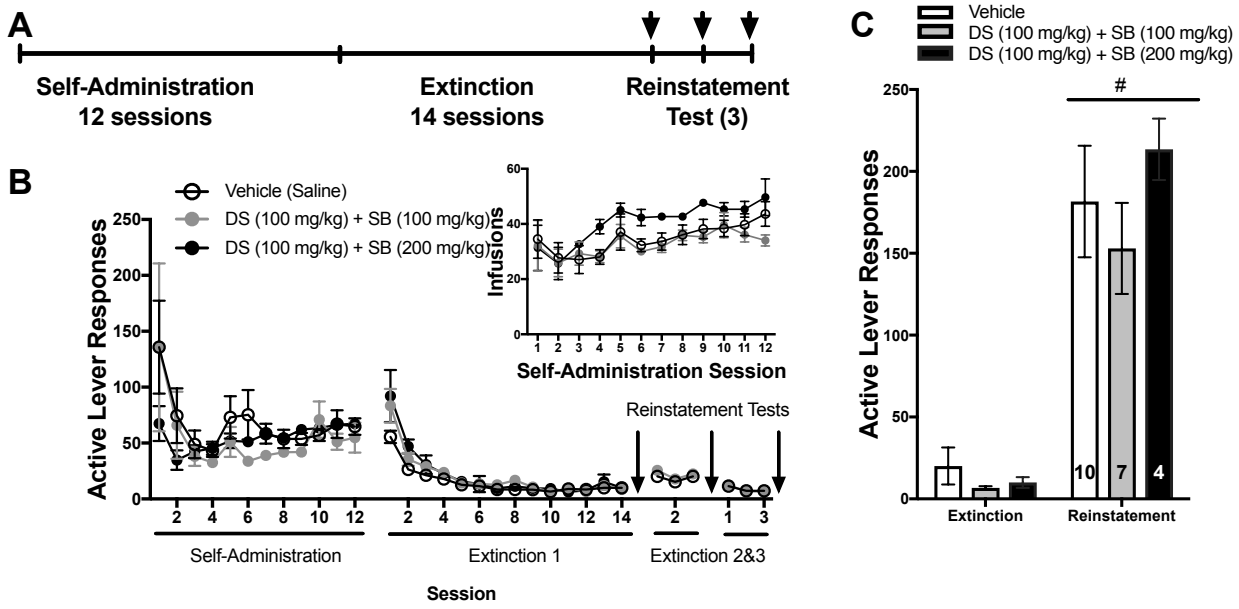


Figure 2.3. **3 Days of D-serine augmentation, but not acute administration, increase levels of D-serine in the nucleus accumbens core.** (A)Study timeline. (B) There were no differences between treatment groups in self-administration or extinction training. (C) 3-Day, but not acute, D-serine augmentation significantly elevated D-serine levels in the nucleus accumbens core, but not levels of (D) kynurenic acid or (E) kynurenine. Asterisk denotes significant effect *vs.* vehicle (*p<0.05)

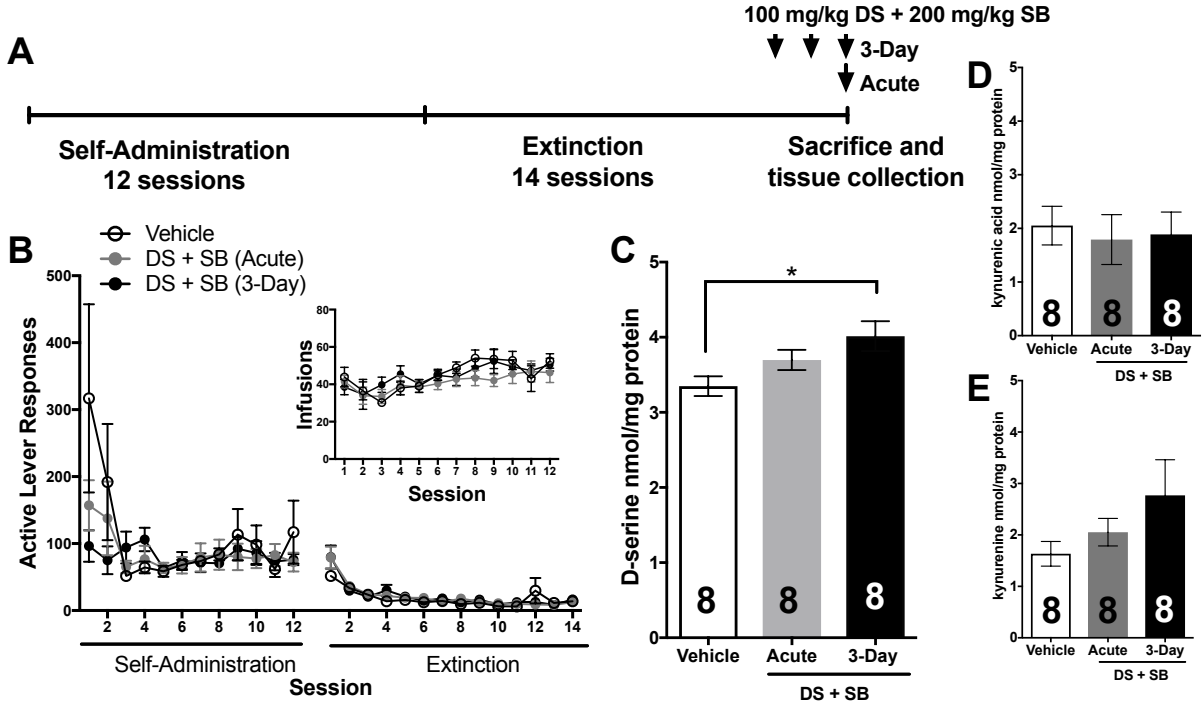


Figure 2.4. D-serine augmentation attenuates cocaine seeking in a model of forced abstinence, but had no effect on locomotor behavior. (A)Study timeline. (B & C) There were no differences between the treatment groups in self-administration training. (D) D-serine augmentation (100 mg/kg D-serine and 200 mg/kg sodium benzoate) significantly attenuated cocaine seeking in a cocaine plus cue-primed extinction test following 14 days of forced abstinence. (E) There were no differences in locomotor activity in animals treated with D-serine (100 mg/kg) and SB (200 mg/kg). Asterisk denotes significant effect *vs.* vehicle (*p<0.05).

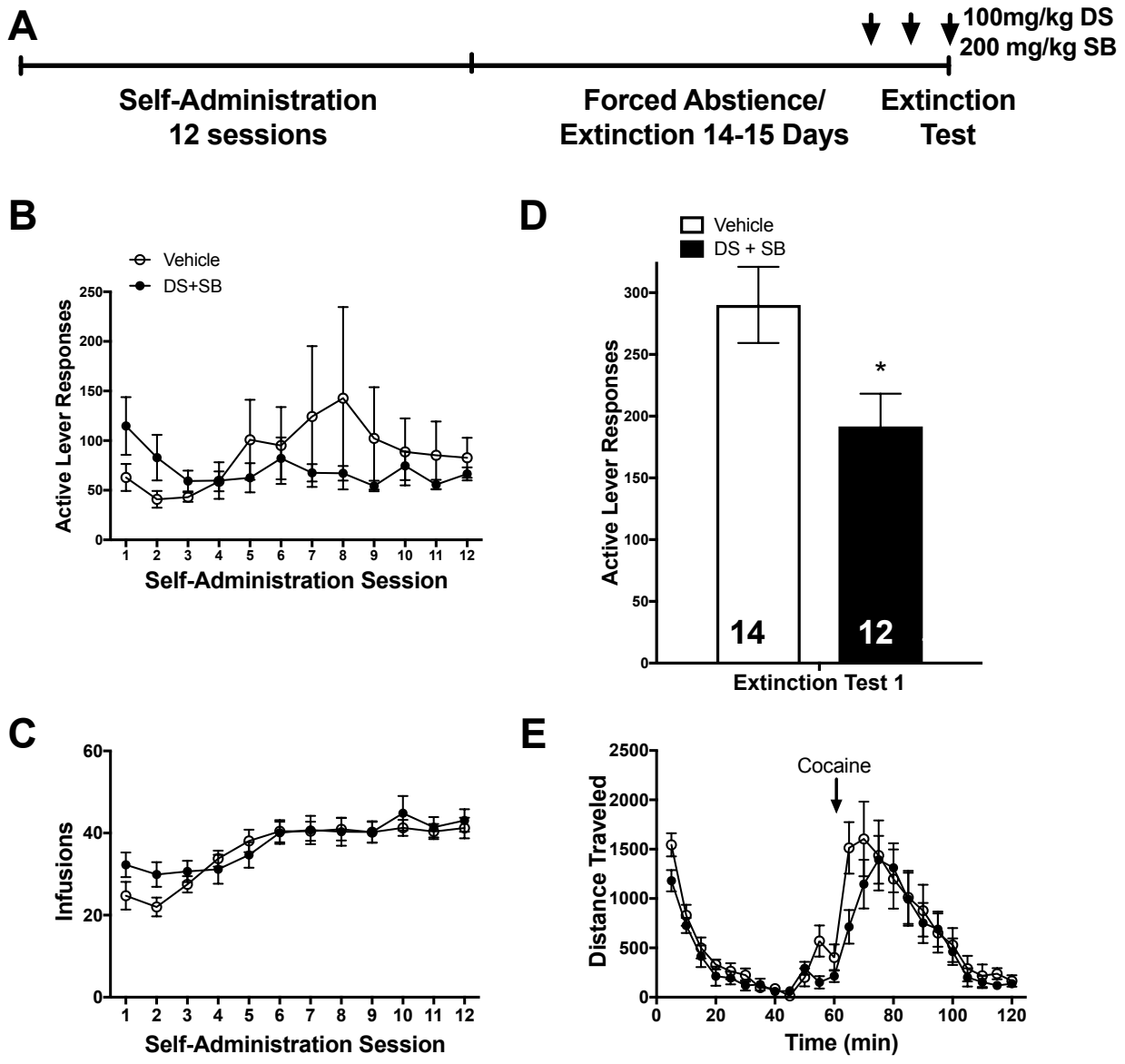
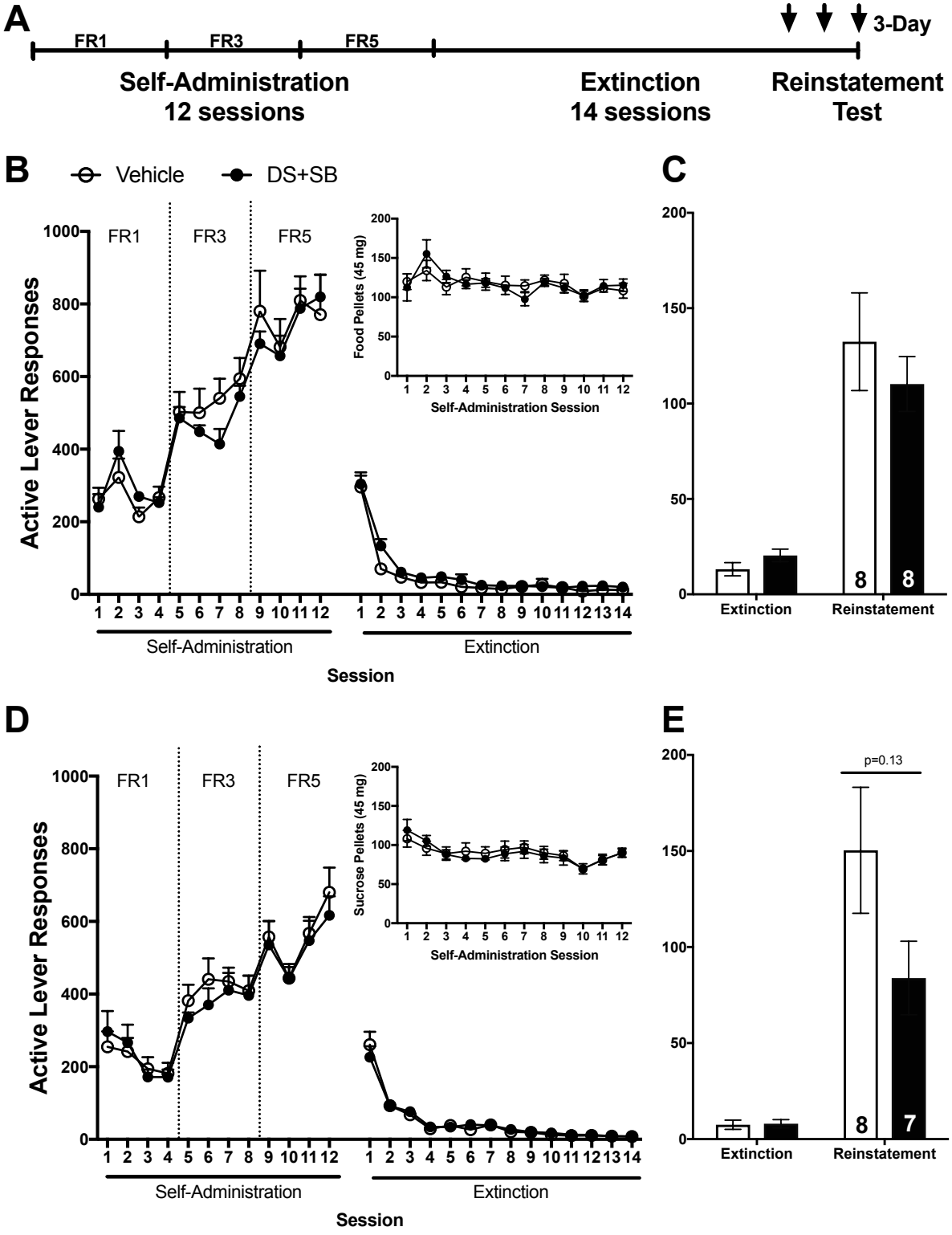


Figure 2.5. D-serine augmentation has no effect on natural reward seeking. (A) Timeline of sucrose and food reinstatement. Self-administration and extinction training responding for (B) food and (D) sucrose was similar between the treatment groups. D-serine augmentation had no effect on reinstatement to (C) food- and (E) sucrose-seeking.



CHAPTER 3:

The Role of the Nucleus Accumbens in the Effects of D-serine Augmentation.

Introduction.

Data presented in the previous chapter indicates that systemic administration of D-serine and sodium benzoate can reduce cocaine reinstatement. These results raise an important subsequent question regarding the mechanism of this effect. I hypothesize that the attenuation in cocaine seeking is mediated by cellular consequences of NMDA receptor stimulation in the NAc. The central role of the NAc in cocaine seeking has been well defined, particularly regarding the alterations in glutamatergic homeostasis and synaptic function following prolonged withdrawal (Kalivas et al., 2003; Scofield & Kalivas, 2014). After cocaine self-administration and extinction training, glutamate transporters (GLT-1 and system xc-) are downregulated, resulting in increased synaptic glutamate and glutamate spillover into the extra synaptic space during a reinstatement event (McFarland et al., 2003). This increased excitatory synaptic drive in the NAc is associated with increased AMPA:NMDA ratio, and is believed to contribute to cocaine seeking behaviors characteristic of reinstatement and relapse (Gipson et al., 2013; Shen et al., 2014).

Increases in AMPA:NMDA are often driven by increases in AMPA current, but not always. This is the case following withdrawal from cocaine self-administration, as pharmacological inhibition of AMPA receptors blocks reinstatement of cocaine seeking, and increased surface expression of AMPA receptors have been reported in cocaine withdrawn animals (Wolf & Tseng, 2012). However, it is possible that reduced tone on NMDA receptors could also be adding to the potentiation of the synapse as measured by the AMPA:NMDA ratio. The role for NMDA

receptors in cocaine seeking is murky at best (for review, see (P. I. Ortinski, 2014)), but in general there are reports of increased NMDA GluN1 subunit in the NAc following extended withdrawal from cocaine self-administration (Hafenbreidel et al., 2014; Lu et al., 2003; P. I. Ortinski, 2014; Pomierny-Chamiolo et al., 2015; Tang et al., 2004).

Despite NMDA receptors being present, and possibly upregulated at the surface, I hypothesize that reduced *activation* of NMDA receptors, due to insufficient levels of astrocyte-derived D-serine, a necessary co-agonist of the NMDA receptor. This is supported by findings from our lab that synaptic colocalization of astrocyte peripheral processes in the NAc are reduced in cocaine-extinguished rats (Scofield, Li, et al., 2016). Further, NMDA receptor activation can induce AMPA receptor internalization (Biou et al., 2008; Casimiro et al., 2011) and D-serine has been shown to enhance fear conditioning by increasing AMPA internalization (Bai et al., 2014). Therefore, one possibility for the behavioral effect of D-serine augmentation on reinstatement is increased activation of NMDA receptors leading to reduced surface expression of AMPA receptors, as well as normalized synaptic strength as measured by AMPA:NMDA.

The ability of NMDA receptor stimulation to result in AMPA receptor internalization has been well described in other neural paradigms (Bai et al., 2014; Biou et al., 2008; Casimiro et al., 2011). Particularly, activation of NMDA receptors can induce endocytosis of GluA2-containing AMPA receptors (Casimiro et al., 2011; Luscher et al., 1999). Therefore, stimulation of NMDA receptor function with D-serine would not only result in restored basal synaptic strength, but could also lead to an internalization of GluA2-containing AMPA receptors that are upregulated following prolonged withdrawal (Ma et al., 2014). NMDA receptor stimulation with systemic D-serine augmentation may lead to AMPA receptor endocytosis, normalizing synaptic strength to control levels and blocking the transient synaptic potentiation observed during reinstatement (Gipson et al., 2013; Shen et al., 2014). This mechanism has been more extensively studied as it pertains to LTD, and as such NMDA receptor-induced AMPA endocytosis has been well

characterized in the hippocampus (Biou et al., 2008; T. C. Brown et al., 2005; Casimiro et al., 2011; Migues et al., 2016). It is important to note that NMDA receptor-induced endocytosis of GluA2-contiaining AMPAR does not disrupt constitutive cycling of AMPA receptors in and out of the membrane, only regulated (stimulated) endocytosis (Ahmadian et al., 2004).

Several studies have begun to elucidate the relationship between AMPA receptor endocytosis and cocaine seeking behavior in rodents. Increased surface expression, through disruption of endocytosis and receptor cycling, of both GluA2-lacking (calcium permeable) and GluA2-containing (calcium impermeable) AMPA receptors potentiates reinstatement (Briand, Deutschmann, Ellis, & Fosnocht, 2016; Briand et al., 2014; Schmidt et al., 2015), and disrupting stabilization in the membrane attenuates cocaine seeking (James et al., 2014; S. L. White et al., 2016). It is important to note that some studies have found conflicting results for AMPA trafficking and drugs of abuse. For example, increased surface expression of GluA2-lacking AMPA receptors was found to attenuate cocaine primed reinstatement to cocaine seeking, but had no effect on cue-induced reinstatement (Bachtell & Self, 2008). Another study found that disrupting trafficking of GluA2 containing AMPA receptors to and from the surface attenuates cue-induced cocaine seeking (Famous et al., 2008). Additionally, conflicting results of GluA2 endocytosis and behavioral sensitization of the psychostimulant amphetamine have been reported; where GluA2 endocytosis has been to found to attenuate behavioral sensitization (Brebner et al., 2005) but also to have no effect on induction or maintenance of amphetamine sensitization (Choi, Ahn, Wang, & Phillips, 2014). While the collective interpretation of the literature is somewhat complex, overall it is agreed that withdrawal from cocaine leads to increased AMPA activity, and that inhibition of AMPA receptors impairs drug seeking.

Although the effects of cocaine on AMPA receptor activity have been well researched, NMDA receptor mediated endocytosis of GluA2 containing AMPA receptors has yet to be investigated in the NAc or in the context of NAc drug-mediated behaviors. Targeting NMDA receptor-mediated internalization may normalize increased AMPA receptor expression and/or

activity that results in potentiated MSN synapses in the NAc core and subsequently decrease drug seeking. Evidence from other studies indicate that D-serine stimulation of NMDA receptors can lead to GluA2 containing AMPA receptor internalization (Bai et al., 2014; Kakegawa et al., 2011) and synaptic depotentiation and/or depression in cocaine withdrawn rats or in the supra optic nucleus of lactating rats (Curcio et al., 2013; Panatier et al., 2006). If D-serine augmentation attenuates cocaine seeking by depotentiating the synapse through NMDA receptor activation and AMPA receptor internalization, surface expression of AMPA receptors will be reduced in D-serine augmented animals.

Methods.

Animals.

Male Sprague Dawley rats (Harlan Farms, Raleigh NC), aged approximately 6-8 weeks and weighing 260-300 grams at the time of surgery were used in these experiments. Rats were individually housed in a temperature controlled environment on a reversed 12:12 light:dark (lights off at 0700) schedule. Following a week of habituation, animals were put on a food restricted diet of 20g/day (Envigo Teklad laboratory animal diet). The University of North Carolina's Institutional Animal Care and Use Committee approved all procedures.

Catheterization surgery and stereotaxic surgery.

Chronic indwelling catheters were implanted into the right jugular vein for the administration of intravenous (*iv*) cocaine. Catheters were created as described previously (Scofield, Li, et al., 2016) and exited the animals' back between the scapulae. In some experiments, following catheterization animals were moved to the stereotaxic frame (David Kopf Instruments; Tujunga, CA) and surgically implanted with bilateral 26-gauge guide cannula (Plastics One; Roanoke, VA) in the NAc core (A/P: 1.4 mm, M/L: 1.7mm, D/V: -5.5 mm). Animals received 5 days of recovery and catheters were flushed daily with antibiotic (gentimicin

5mg/ml, 0.1 ml, *iv*) followed by heparinized saline (100 mg/ml, 0.1 ml, *iv*) following surgery during recovery and throughout self-administration training.

Behavioral training.

Self-Administration and Extinction Training.

Self-administration training was conducted in standard operant conditioning chambers (Med Associates Inc.) that contained a white houselight, two retractable levers, and a bar floor. Rats were first trained to lever press for food in a food training session where criteria was set at 6 hr or 100 pellets delivered. After recovery from surgery, animals were trained to lever press for cocaine infusions (cocaine hydrochloride; 0.2 mg per infusion, *iv*; NIDA) under a fixed ratio (FR1) reinforcement schedule with a discrete stimulus complex during infusion and 20*s* time out. The stimulus complex consisted of a tone (70 dB, 2.5 kHz) and a stimulus light above the active lever that activated for 5 *s* during the infusion. There was a second lever that had no programmed response when pressed; this served as a control for lever responding throughout the experiment. Rats were trained to self-administer cocaine in 2 hr daily sessions for 12 days or until they achieve the acquisition criterion (10 days of greater than 10 infusions per session). Treatment groups were balanced on the last 3 days of self-administration active lever presses. Following self-administration animals received extinction training where responding on either lever had no programmed result. Rats received 14 extinction training sessions before the test of reinstatement.

Tests of Reinstatement.

Reinstatement to cocaine seeking was defined as a significant increase in active lever responding on the test day relative to responding during the last extinction training session. Twenty-four hr after the last extinction training session rats were tested for reinstatement of cocaine-seeking behaviors in a 1 or 2 hr cocaine plus cue-primed test session. Directly before

being placed in the chamber, rats received a cocaine prime (10 mg/kg, *i.p.*). During the test session, lever responses were recorded without cocaine reinforcement. The stimulus complex was presented identical to self-administration training on lever press of the active lever on an FR1 schedule (cue prime). Inactive lever responses were recorded but had no programmed response.

Locomotor Test.

Rats were tested for locomotor activity of habituation to a novel environment and a cocaine locomotor challenge. Standard locomotor chambers (Med Associates Inc.) with beams to track locomotor activity were used. Rats were placed in the center of the chamber and the 2 hr locomotor test began. 60 minutes into the locomotor test the session was paused for a cocaine injection (10 mg/kg, *i.p.*). Locomotor activity was collected in 5 minute bins in mm of ambulatory activity.

Treatment.

Systemic

Rats received 3 days of D-serine augmentation treatment that consisted of D-serine and sodium benzoate (SB; DAO inhibitor). D-serine augmentation was administered systemically 3 hr prior to the last two extinction sessions (100 mg/kg D-serine*, i.p.*; 100 mg/kg SB, *i.p.*) and on reinstatement or extinction test day (100 mg/kg D-serine, *i.p.*; 200 mg/kg SB, *i.p.*).

Nucleus Accumbens

Rats with cannula aimed at the NAc core received 3 days of NMDA receptor stimulation. Preliminary experiments (not shown) indicated that direct intra-NAc administration of SB can lead to neural toxicity, and accordingly it was not used as in the systemic studies. Instead, D-serine (100 μg and 300 μg in 1 μL) alone was administered directly into the NAc 20 min prior to the last two extinction sessions, as well as 20 min prior to the reinstatement test. In some

groups, NMDA, an agonist of the NMDA receptor, was microinjected into the NAc core alone or with the low dose D-serine (100 μg/μL/side) only on the day of reinstatement test. Cocaine self-administering animals received the following treatments: vehicle (veh; 0.9% NaCl), D-serine (100 μg/μL/side and 300 ug/μL/side), NMDA (0.25 μg/μL/side), and D-serine + NMDA (3-days 100 μg/μL/side and 1 day 0.25 μg/μL/side, respectively).

Surface and total expression of AMPA and NMDA receptor subunits.

In order to test protein trafficking during the test of reinstatement two time points for rapid decapitation were set. The first was 3 hrs after the third treatment (directly before the test of reinstatement would occur) and the second was 1 hr into the cocaine plus cue-primed reinstatement test. Both treatments were tested at each time point. Rats in the reinstatement test group received a cocaine prime (10 mg/kg) directly before being placed in the test session. Transient potentiation of NAc core synapses following cue-primed reinstatement has been shown to return to basal levels by 120 minutes following cue-prime (Gipson et al., 2013), and so reinstatement test (see Chapter 2 for detailed methods) was limited to 1 hr in this experiment. Following a 1 hr reinstatement test, or 3 hrs post injection for no reinstatement groups (t=0), rats were decapitated and NAc core, dorsal hippocampus and dorsolateral striatal tissue were taken for surface biotinylation and Western blot analysis for AMPA receptor subunits GluA1 and GluA2, NMDA receptor subunits GluN1, GluN2A and GluN2B and control protein GLT-1 as described (Reissner et al., 2011).

Procedures followed (Reissner et al., 2011). NAc core, dorsal hippocampus and dorsolateral striatum tissue were dissected (2 mm punches of NAc core and dorsolateral striatum, dorsal hippocampus was free hand dissected) and chopped (Mackelwain tissue chopper) followed by a 30 min incubation in PBS containing 1mg/ml Sulfo-NHS-Biotin at 4ºC with gentle shaking. After incubation, to quench the biotinylation reaction, the tissue was

washed twice in ice-cold 100 mM glycine in PBS. Tissue was then sonicated in 1% SDS in RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific). The lysate was centrifuged at 10,000 x g for 10 min at 4ºC, and the supernatant was taken for protein determination by the BCA method (Thermo Scientific). 100 μg of the supernatant was combined with NeutrAvidin agarose resin and incubated overnight at 4ºC with gentle rotation. After two washes of ice-cold PBS, biotinylated proteins were eluted in 1% SDS, 50 mM DTT loading buffer and heated to 90 ºC for 5 min. Proteins in the biotinylated and total protein fractions were detected by immunoblotting.

Western Blot

10 μg of protein from the total protein fraction or 15 μl of biotinylated fraction were separated per lane on 7.5% Criterion Tris-HCl gels (Bio-Rad; 180mV, ~45 min) and transferred 1hr at 150 mA onto PVDF membranes. Membranes were dried and then stained for total protein content using REVERT Total Protein Stain (LICOR Biosciences). Membranes were then blocked for 1hr at room temperature in LICOR Odyssy Tris blocking solution and incubated with primary antibodies overnight at 4°C (GluA1, Abcam ab31232, 1:1000; GluA2 Millipore MABN71, 1:2000; GluN1, Millipore, 05-432, 1:600; GluN2A, Millipore MAB5530, 1:1000; GluN2B, Abcam ab65783, 1:1000; GLT-1, Millipore, AB1783, 1:5,000). Secondary antibody incubation was performed for 1.5hr at room temperature (700RD anti-rabbit, and 800CW anti-mouse, 800CW anti-mouse, 700RD anti-Guinea pig, LICOR Bioscience, 1:15,000 each). Westerns were imaged on an LICOR Odyssey Fc imager. Proteins of interest were adjusted to protein content in each lane using REVERT total protein stain at either 150-250 kDa or 75-100 kDa, as applicable, (LICOR Bioscience) and normalized to saline (vehicle) T=0 control.

Data analysis.

A 2x2 repeated measures analysis of variance (ANOVA) was used to compare the behavioral data of the groups (active lever responding, inactive lever responding and infusions). A 2x2 ANOVA was used to investigate differences in protein expression (time point x treatment) with Sidak's multiple comparison's test *post hoc* corrections to compare groups.

Results.

NAc core D-serine augmentation attenuates cue- and drug-primed test of reinstatement.

Experiment 1 was designed to determine whether NMDA receptor stimulation via D-serine administration specifically in the NAc is sufficient to inhibit cocaine reinstatement, akin to systemic D-serine augmentation. Animals were trained in cocaine self-administration followed by extinction and a cocaine plus cue-primed reinstatement test. Intra-NAc D-serine was administered 20 min prior to the last two extinction session, and D-serine plus NMDA was administered 20 min prior to the reinstatement test. There were no differences observed between treatment groups in active lever responding ($F_{(4.33)}$=0.5, p=0.72) or cocaine infusions ($F_{(4.33)}$=0.9, p=0.50) during self-administration training, and all treatment groups extinguished active lever responding during extinction training (ME of session $F_{(13.429)}$=6.4, p<0.01, Sidak's multiple comparisons test, $p<0.05$) with no differences between the groups ($F_{(4.33)}$=1.7, p=0.17). Table 3.1 details the full statistics of the training behavior data. Test session cocaine seeking was compared with the last day of extinction training to determine reinstatement to cocaine seeking and any treatment effects. All groups significantly increased responding during reinstatement compared to extinction responding (IE $F_{(4,41)}$=2.6, p<0.05; ME of Session $F_{(1,41)}$=117.8, p<0.01) and there was a significant effect of treatment (ME of Treatment $F_{(4,41)}$=2.6, p=0.05; *post hoc* Sidak's test of multiple comparisons p<0.05). *Post hoc* analyses revealed there was no significant effect of either dose of D-serine alone (300 μg/side, 100 μg/side; Figure 3.1). Also, NMDA alone (0.25 μg/side) did not significantly affect reinstatement behavior,

however in combination with a low dose of D-serine (100 μg/side), cocaine seeking was significantly attenuated (Figure 3.1D). Thus, a sub-threshold dose of either D-serine or NMDA when administered together is sufficient to impair reinstatement, supporting the hypothesis that D-serine is mediating its effects through the NMDA receptor.

Intra-accumbal injection of D-serine and NMDA had no effect on locomotor activity (Figure 3.1E). Animals habituated to the novel environment and increased activity following a cocaine challenge (IE $F_{(23.322)}$=0.7, p=0.86; ME of Time $F_{(23.322)}$=23.58, p<0.01), yet there were no differences between the groups in locomotor activity ($F_{(1,14)}$=0,4842, p>0.05).

D-serine augmentation does not alter AMPA or NMDA surface expression in the nucleus accumbens core.

To determine the mechanism by which D-serine augmentation is affecting reinstatement of cocaine seeking, we explored changes in AMPA and NMDA receptor subunits and the glutamate transporter GLT-1 total protein and surface protein expression in the NAc core, hippocampus and striatum at two time points. Immunoblotting targeted AMPA receptor subunits GluA1 and GluA2, and NMDA receptor subunits GluN1, GluN2A and GluN2B.

Behavior Data

Responding during self-administration and extinction training (Table 3.1) was not different between the groups (p>0.05, Figure 3.1). A subgroup of animals received a cocaine plus cue-primed reinstatement test. D-serine augmented and vehicle-treated animals significantly increased responding during reinstatement test compared to extinction responding (IE $F_{(1,19)}$=1.8, p=0.19; ME of Session $F_{(1,19)}$=93.5, p<0.01; Figure 3.2B), however, there was no effect of treatment on cocaine seeking (ME of Treatment $F_{(1,19)}$=1.9, p=0.18; Figure 3.2C).

Nucleus Accumbens Core Protein Expression

NMDA receptor subunit GluN1 total protein expression was increased in D-serine augmented animals (IE $F_{(1,38)}$=1.7, p=0.20; ME of Treatment $F_{(1,38)}$=10.6, p<0.005; Figure 3.3A)

and *post hoc* analysis revealed that basal levels of GluN1 (T=0) D-serine augmented animals were significantly increased compared to vehicle animals (Sidak's test of multiple comparisons, $p<0.05$). There were no differences between the treatment groups, or between basal and reinstatement levels of NMDA receptor subunits GluN2A or GluN2B (Figure 3.3B-C) and no changes in surface expression of any of the NMDA receptor subunits (Figure 3.5A-C).

AMPA receptor subunit GluA1 total expression had a significant interaction ($F_{(1,39)}=6.0$, $p<0.02$; Figure 3.4A), and *post hoc* analyses divulged that reinstatement significantly increased GluA1 levels in vehicle treated animals; however, D-serine augmented animals remained at basal levels. GluA2 levels were unchanged overall or at the surface at either time point, and GluA1 levels were unchanged at the surface as well.

GLT-1 expression was altered following reinstatement test in the NAc, in total expression and surface expression (Figure 3.7A-B). A 1 hr cocaine plus cue-primed reinstatement test increased GLT-1 total protein expression (IE $F_{(1,39)}=0.02$, p=0.90; ME of Time $F_{(1,39)}=4.8$, $p<0.05$), however no *post hoc* analyses were significant between the treatments. Mirroring this, an increase in surface expressed GLT-1 was also seen following cocaine plus cue-primed reinstatement test. NAc core GLT-1 surface expression had a significant interaction effect ($F_{(1,39)}=7.1$, $p<0.02$), and *post hoc* analysis revealed a significant increase in GLT-1 surface expression in vehicle treated animals after 1 hr of the reinstatement test compared to basal levels. Further investigation of this effect indicated that the upregulation of GLT-1 surface expression was driven by a significant reduction in total protein in the 1 hr reinstatement group between the molecular weights of 75-100 kDa. REVERT total protein stain (normalized to saline T=0 control) was significantly reduced in vehicle animals after the 1 hr reinstatement test compared to basal levels (IE $F_{(1,81)}=9.9$, $p<0.01$; Sidak's multiple comparison vehicle T=0>T=1, $p<0.01$), and as the total protein stain is used to adjust GLT-1 signal by dividing GLT-1 signal with REVERT total protein signal, this could be driving the decrease in GLT-1 surface expression. Additionally, NAc core REVERT total protein signal was significantly different

between the treatments at T=0 (IE $F_{(1,82)}$=2.7, p=0.21; ME treatment $F_{(1,82)}$=4.0, p<0.05; Sidak's multiple comparison vehicle T=0 > treatment T=0, p<0.05). This may be masking an even greater effect of GluN1 increase in D-serine augmented animals. REVERT total protein stain signal was not significantly different in the biotinylated fraction or total protein fraction of the other brain areas. These results need to be further investigated to decipher if a GLT-1 signal is upregulated, or a mass surface internalization event is causing a decrease in total protein of vehicle treated reinstated animals.

Dorsolateral Striatum and Dorsal Hippocampus Protein Expression Protein Expression.

The effects of reinstatement and D-serine augmentation were also investigated in the dorsolateral striatum and dorsal hippocampus. In the striatum, an anatomical control region, total and surface expression of AMPA (Figure 3.3) and NMDA (Figure 3.4) receptors was similar between groups, indicating D-serine augmentation had no effect on receptor subunit protein translation, transcription or trafficking. Striatal GLT-1 total protein expression was affected by reinstatement test (IE $F_{(1,39)}$=0.3, p<0.60; ME of Time $F_{(1,39)}$=6.9, p<0.02) and *post hoc* analysis revealed a trend for vehicle animals to have increased GLT-1 protein following a 1 hr reinstatement test (Sidak's multiple comparison, p=0.07; Figure 3.7E). There were no differences in striatal surface expression of GLT-1 (Figure 3.7F).

The dorsal hippocampus was investigated because D-serine facilitates cocaine extinction learning (H. Han et al., 2015; Kelamangalath et al., 2009; Kelamangalath & Wagner, 2010), and the hippocampus is a structure important in learning and memory. Hippocampal protein levels did not change between treatment groups at either time point in overall protein or surface protein expression for AMPA (Figure 3.4C&D; Figure 3.6E&F) or NMDA (Figure 3.4D-F; Figure 3.G-I) receptor subunits or GLT-1 (Figure 3.7C&D).

Discussion.

Contributions of cocaine-induced synaptic modifications to cocaine seeking behaviors

Impaired synaptic function in the NAc core has been well described in cocaine withdrawn animals, and the seminal features are impaired glutamate homeostasis and potentiated synaptic strength (Carbone et al., 2012; Gipson et al., 2013; Kalivas, 2009; Kalivas et al., 2009; Shen et al., 2014). We have recently included decreased synaptic colocalization of astrocytic processes to these sequelae of changes (Scofield, Li, et al., 2016). I hypothesize that decreased synaptic colocalization will increase the barrier to volume transmission of astrocyte-derived factors, including D-serine, and consequentially reduce NMDA receptor function contributing to the synaptic adaptations induced by cocaine withdrawal. Supporting this, treatment with ceftriaxone restores synaptic colocalization of astrocyte processes after cocaine self-administration and extinction (Scofield, Li, et al., 2016) and blocks cocaine reinstatement (Knackstedt et al., 2010). Further, I have found that direct NMDA receptor stimulation in the NAc core attenuates cocaine seeking (Chapters 2 and 3). Collectively, these results suggest that rectifying the maladaptive cocaine-induced changes to astrocytes can reduce reinstatement to cocaine seeking, and more specifically, that stimulation of NAc core NMDA receptors can reduce reinstatement.

Our recent finding that astrocytes retract from the synapse following cocaine self-administration and extinction (Scofield & Kalivas, 2014), may indicate a reduction in the ability to activate NMDA receptors due to limited volume transmission of its co-agonist D-serine. This is in agreement with previous reports of impaired NMDA receptor function following cocaine experience (Curcio et al., 2013), but is opposed to reports regarding increased expression of NMDA receptors following cocaine self-administration and extinction (Hafenbreidel et al., 2014). Importantly, behavioral results presented herein specifically regard stimulation of NMDA receptors, independent of expression levels. It is possible that increased subunit expression may be a compensatory effort by the cell to normalize NMDA receptor function.

The inability of direct D-serine administration alone into the NAc core at a higher dose (300 μg/side) to attenuate cocaine seeking is at odds with our hypothesis that D-serine and SB administered together increase D-serine levels driving NMDA receptor activation and reducing reinstatement. This could be an indication that the NAc core is not the only brain region driving the systemic D-serine augmentation effect or that DAO acting on other molecules (for instance kynurenic acid) are driving our effect. However, it is unlikely that DAO inhibiting the production of kynurenic acid is the mechanism driving our systemic effect, as we found no differences in kynurenine or kynurenic acid following 3 days of systemic D-serine augmentation in the NAc core. Previous studies have found that administration of SB to the VTA increases dopamine release in the mPFC (Betts et al., 2014), and also that NMDA administration to the NAc increases mPFC glutamate (Bortz, Wu, Schwarcz, & Bruno, 2017), potentially indicating that both administration to the NAc and administration of systemic D-serine augmentation are activating the mPFC, an area of descending input that regulates motivation (McGlinchey et al., 2016; Perry et al., 2011). In fact, PFC-subcortical circuits are integral for inhibitory control over cocaine craving and seeking (Navailles, Guillem, Vouillac-Mendoza, & Ahmed, 2015). Supporting this, systemic and intra-NAc D-serine reduces aversion resistant alcohol intake, but not quinine free-alcohol (Seif et al., 2015). Additionally, ventral medial PFC recruitment of glycine and D-serine were blunted after protracted abstinence from long-term alcohol exposure and, further, administration of a glycine transport inhibitor attenuated motor impulsivity deficits of alcohol exposed animals (Irimia et al., 2017). The short access and extinction model of cocaine administration used in these experiments does not have adverse consequences, such as models of inhibitory control or model deficits in impulsivity, and as such it is generally accepted that short access and extinction paradigms access the motivational properties of the drug and not inhibitory control. Despite this, it is an interesting possibility that should be further investigated.

Possible roles for NMDA receptor subtypes in cocaine reinstatement

Collectively, our total protein expression data indicates divergent effects of D-serine augmentation on the obligatory subunits of AMPA and NMDA receptors, GluA1 and GluN1, respectively. D-serine augmentation increases total protein expression of the NMDA receptor subunit GluN1, where total GluN1 protein expression is significantly greater than vehicle treated animals basally (T=0), but these trends to do not follow to surface expression, which was unchanged. GluN1 is an obligatory subunit in the NMDA receptor complex, and as such an increase in GluN1 expression could be indicative of an overall increase in NMDA receptors following cocaine self-administration and prolonged withdrawal. The NMDA receptor complex is a heterotetramer, where one GluN1 dimer is necessary, but the second dimer can be composed of several match pairs of NMDA receptor subunits. Importantly, GluN1 contains the glycine modulatory site for binding of D-serine or glycine. The next two most common NMDA receptor subunits are GluN2A and GluN2B, which were assessed in these experiments. NMDA receptor subunits GluN2A-D bind glutamate (Furukawa, Singh, Mancusso, & Gouaux, 2005; Monyer et al., 1992). GluN3 subunits also contain the GMS and bind D-serine and glycine, and have been shown to have exponentially greater binding of the co-agonists than the NR1 subunit (Yao & Mayer, 2006). Interestingly, it has been recently found that the cocaine-evoked plasticity at VTA dopamine neurons that synapse with excitatory projections is caused by insertion of GluN3A-containing NMDA receptors (Yuan et al., 2013). Because these subunits, such as GluN2C/D or GluN3A were not targeted in my experiments, it is possible that these were also upregulated with GluN1 to create more NMDA receptors to be trafficked to the surface.

Additionally, a reinstatement event induced increased expression of the AMPA receptor subunit GluA1 in vehicle treated animals, but this upregulation of protein is not seen in D-serine augmented animals, indicating that D-serine augmentation is blocking this effect. As surface expression remained unchanged for all subunits of AMPA and NMDA receptors, it is unlikely that these changes in total protein are the mechanism by which D-serine augmentation is

attenuating reinstatement. They do, however, give insight into the effects of cocaine plus cue-primed reinstatement on protein expression, and how D-serine augmentation affects these reinstatement-induced changes in protein.

Given our surface expression data, it is unlikely that in the NAc D-serine augmentation is inducing AMPA receptor internalization, and through AMPA receptor internalization depotentiating the synapse and attenuating cocaine seeking. This leads us to contemplate other mechanisms by which NMDA receptor activation is reducing cocaine seeking. One alternative is NMDA receptor mediated dopamine release inhibition.

Potential mechanism of the effect of D-serine on reinstatement

As stated, cocaine exposure profoundly affects AMPAR transmission in the NAc core and shell, and similar changes also occur in the ventral tegmental area (VTA), the origin of the mesolimbic dopamine system. In the VTA a single cocaine injection can initiate exchange if GluA2-containing AMPA for GluA2-lacking (calcium permeable) AMPA receptors, a change which returns to baseline one week later (Bellone & Luscher, 2006). In fact, drugs that activate dopamine release have been shown to cause similar AMPA receptor redistribution in the VTA (Brown et al., 2010). Further, cocaine has been shown to cause a redistribution of NMDA receptors at excitatory synapses projecting on VTA dopamine neurons, inserting GluN3A containing NMDA receptors and effectively reducing NMDA receptor function (Yuan et al.). Adding insight into the decreased AMPA/NMDA ratio observed following cocaine exposure, as not only being a result of an increased AMPA receptor signal, but a decreased NMDA receptor-mediated component (Mameli, Bellone, Brown, & Luscher, 2011; Shen et al., 2014). As a result of these finds, the mesolimbic dopamine system is proposed as a point of convergence for addictive drugs to alter neural circuits.

The AMPA receptor exchange of GluA2-containing for GluA2-lacking AMPA receptors has also been well characterized in the NAc shell (Wolfe et al. 2012), however these changes

are not present in the NAc core (Ma et al., 2014). Despite this, nucleus accumbens dopaminergic and glutamatergic projections synapse on the same spines of medium spiny GABAergic projection neurons (Sesack, Carr, Omelchenko, & Pinto, 2003), making it another potential locus of these alterations in neural circuits. Specifically, the nucleus accumbens core remains to be investigated for alterations in NMDA receptor subunit composition, especially given the ability for direct NMDA receptor activation to attenuate cocaine seeking, shown here. Given the changes in NMDA receptor signaling in the VTA, in part, accounts for the reduction in AMPA:NMDA ratio (Mameli et al., 2011), this is something that also remains to be investigated in the NAc core. Additionally, as mentioned above, we did not probe for changes in GluN2C/D or GluN3A NMDA receptor subunits, which have been shown to be upregulated in the mesolimbic dopamine system following drugs of abuse (Hagino et al., 2010; Yuan et al., 2013) and Parkinson's disease (Feng, Zhang, & Chergui, 2014; X. Zhang, Feng, & Chergui, 2014), a disease characterized by degenerative dopamine levels. Increased levels of GluN2C/D or GluN3A could be reducing the NMDA receptor-mediated currents, and thus our systemic and intra-accumbal targeting of NDMA receptors could be rectifying this deficit and attenuating excitatory signaling in the NAc core. Convergent glutamatergic and dopaminergic afferents in the NAc core have complex interactions, and it is possible that NDMA receptors modulate dopamine release at the terminals of VTA afferents, as in the striatum (H. Zhang & Sulzer, 2012). Dopamine modulates PL-NAc glutamatergic transmission, in a dopamine receptor 2 (D2) dependent manner (W. Wang et al., 2012), and as acute cocaine increases dopamine release in the NAc core, this should be considered. Investigation into the interplay between AMPA and NMDA receptor synaptic changes, as well as their effects on the mesolimbic dopamine release are enticing new directions, however differences in the amount of cocaine exposure and withdrawal periods need to be kept in mind.

D-serine augmentation had no effect on NMDA receptor total protein expression in the dorsal hippocampus or the dorsolateral striatum either basally or directly following a

reinstatement test. Additionally, surface expression at either time point was not altered in these brain regions following D-serine augmentation. Further, AMPA receptors (GluA1 and GluA2 subunits) and the glutamate transporter 1 (GLT-1) total and surface expression were also unchanged in these regions by D-serine augmentation, as expected. These results give strong evidence that the cellular mechanism of D-serine augmentation attenuation of reinstatement behavior is through changes in glutamatergic ionotropic receptor or glutamate transporter surface expression in either the dorsal hippocampus or the dorsolateral striatum.

It's possible that cocaine plus cue-primed reinstatement is increasing production of GLT-1 in the striatum, as there was a main effect of time, but no post hoc comparisons were significant. This could be a result of the relatively short period between our two-time points, and potentially if the reinstatement session was extended to a full 2 hr test there would be a greater increase in production of GLT-1 protein. Similar mechanisms may be affecting the NAc as GLT-1 expression was also increased after reinstatement test compared to basal levels, but no differences in between time points within treatments were obtained. Extending the second-time point to after a 2 hr reinstatement test would allow for greater changes in GLT-1 protein. These increases in GLT-1 protein could be a compensatory mechanism to upregulate the transporter as a response to the increased glutamate release during the relapse event. GLT-1 is responsible for clearance of approximately 90% of the synaptic glutamate, and reduced levels of GLT-1 following cocaine and prolonged withdrawal leave the synapse ill prepared for the flood of glutamate during the reinstatement test. As discussed, it has been well characterized that GLT-1 protein is down regulated following multiple paradigms of cocaine self-administration and withdrawal (Fischer-Smith et al., 2012), however changes in GLT-1 protein expression have not been investigated following reinstatement compared to animals who did not experience a relapse event. Although, it is unlikely that the D-serine induced upregulation of GLT-1 protein at the surface is a true effect, as REVERT total protein stain was significantly decreased, likely driving this effect. Further, investigation is necessary to parse out these findings.

In the literature NAc, striatum and hippocampus all have contradictory findings in regard to cocaine self-administration and NMDA receptor subunit expression compared to naïve animals, where most studies find that there are either no change or an increase in GluN1. In the dorsal striatum, following short withdrawal, it has been reported that there is either no change in H-MK801 (radioactive) binding (Ben-Shahar et al., 2007) or an increase in GluN1(Crespo, Oliva, Ghasemzadeh, Kalivas, & Ambrosio, 2002), and further prolonged withdrawal ameliorated the changes in H-MK801 binding (Ben-Shahar et al., 2007). Like the dorsal striatum, the hippocampus has a lack of information on NMDA receptor makeup following either self-administration or after withdrawal, prompting additional probing of each of these areas (P. I. Ortinski, 2014). One experiment showed NMDA receptors expressed at the surface were increased immediately following both cocaine self-administration and yoked cocaine infusions, but following 10 days of extinction training these levels were returned to cocaine naïve levels (Caffino et al., 2014). This lack of hippocampal evidence for NMDA receptor makeup is lieu of a growing amount of studies indicating the hippocampus is important in underlying cocaine-seeking behaviors (Lasseter, Xie, Ramirez, & Fuchs, 2010; Sun & Rebec, 2003), including unpublished work by the author (Healey master's thesis, 2015).

Summary

Data presented in this chapter collectively indicates that D-serine administration together with subthreshold, low dose NMDA administration directly into the NAc core impairs reinstatement without effect on locomotor activity. However, systemic D-serine augmentation does not affect the surface expression of NMDA receptor subunits GluN1, GluN2A or GluN2B, or AMPA receptor subunits GluA1 or GluA2 in the NAc core, dorsal hippocampus or the dorsolateral striatum. Trafficking of proteins can happen on the time scale of minutes (Biou et al., 2008), and so the 1 hr reinstatement test would be ample time for changes in the surface to be revealed. These findings suggest that stimulation of NMDA receptors in the NAc core is

sufficient to reduce reinstatement, but that internalization of AMPA receptors leading to synaptic depotentiation following NMDA receptor stimulation in the NAc is not likely the mechanisms responsible for the effect of systemic D-serine. Future studies will be required to determine the mechanism of action, as discussed below in Chapter 4.

Table 3.1. Behavioral Data.

Experiment	Behavior	F (interaction)	P	F (Session)	P	F(Treatment)	P
Intra-NAc	Self-Ad Active Lever	F (44, 363) = 0.7745	P=0.8493	F (11, 363) = 1.23	P=0.2651	F (4, 33) = 0.5191	P=0.7222
	Self-Ad Inactive Lever	F (44, 363) = 1.865	P=0.0012	F (11, 363) = 6.543	P<0.0001	F (4, 33) = 2.728	P=0.0458
	Extinction Active Lever	F (52, 429) = 1.846	P=0.0006	F (13, 429) = 96.44	P<0.0001	F (4, 33) = 1.732	P=0.1664
	Extinction Inactive Lever	F (52, 429) = 0.9815	P=0.5140	F (13, 429) = 10.53	P<0.0001	F (4, 33) = 0.9256	P=0.4609
	infusions	F (44, 363) = 0.9405	P=0.5832	F (11, 363) = 17.86	P<0.0001	F (4, 33) = 0.8628	P=0.4964
	Reinstatement	F (4, 41) = 2.607	P=0.0496	F (1, 41) = 117.8	P<0.0001	F (4, 41) = 2.6	P=0.0500
Surface Expression	Self-Ad Active Lever	F (33, 418) = 0.7512	P=0.8411	F (11, 418) = 2.538	P=0.0041	F (3, 38) = 0.5064	P=0.6802
	Self-Ad Inactive Lever	F (33, 429) = 0.5943	P=0.9653	F (11, 429) = 6.423	P<0.0001	F (3, 39) = 0.1406	P=0.9350
	Extinction Active Lever	F (39, 494) = 0.4871	P=0.9965	F (13, 494) = 73.36	P<0.0001	F (3, 38) = 1.522	P=0.2244
	Extinction Inactive Lever	F (39, 507) = 1.133	P=0.2717	F (13, 507) = 27.77	P<0.0001	F (3, 39) = 0.6389	P=0.5945
	infusions	F (33, 429) = 1.154	P=0.2602	F (11, 429) = 24.02	P<0.0001	F (3, 39) = 0.6211	P=0.6056
	Reinstatement	F (1, 19) = 1.836	P=0.1913	F (1, 19) = 93.5	P<0.0001	F (1, 19) = 1.906	P=0.1834

Figure 3.1. D-serine plus NMDA administration into the nucleus accumbens attenuates reinstatement to cocaine seeking in a cocaine plus cue-primed test. (A) Study timeline, microinjections were given on the last 2 days of extinction training and on reinstatement test day. (B) Self-administration and extinction training were similar between the treatment groups. (C) Cannula placement were all located in the nucleus accumbens core. All treatment groups reinstated cocaine seeking behaviors, however only the D-serine and NMDA combination treatment significantly attenuated cocaine seeking compared to vehicle. Hash denotes significant effect of reinstatement (#p<0.05). Asterisk denotes significant effect *vs.* vehicle (*p<0.05).

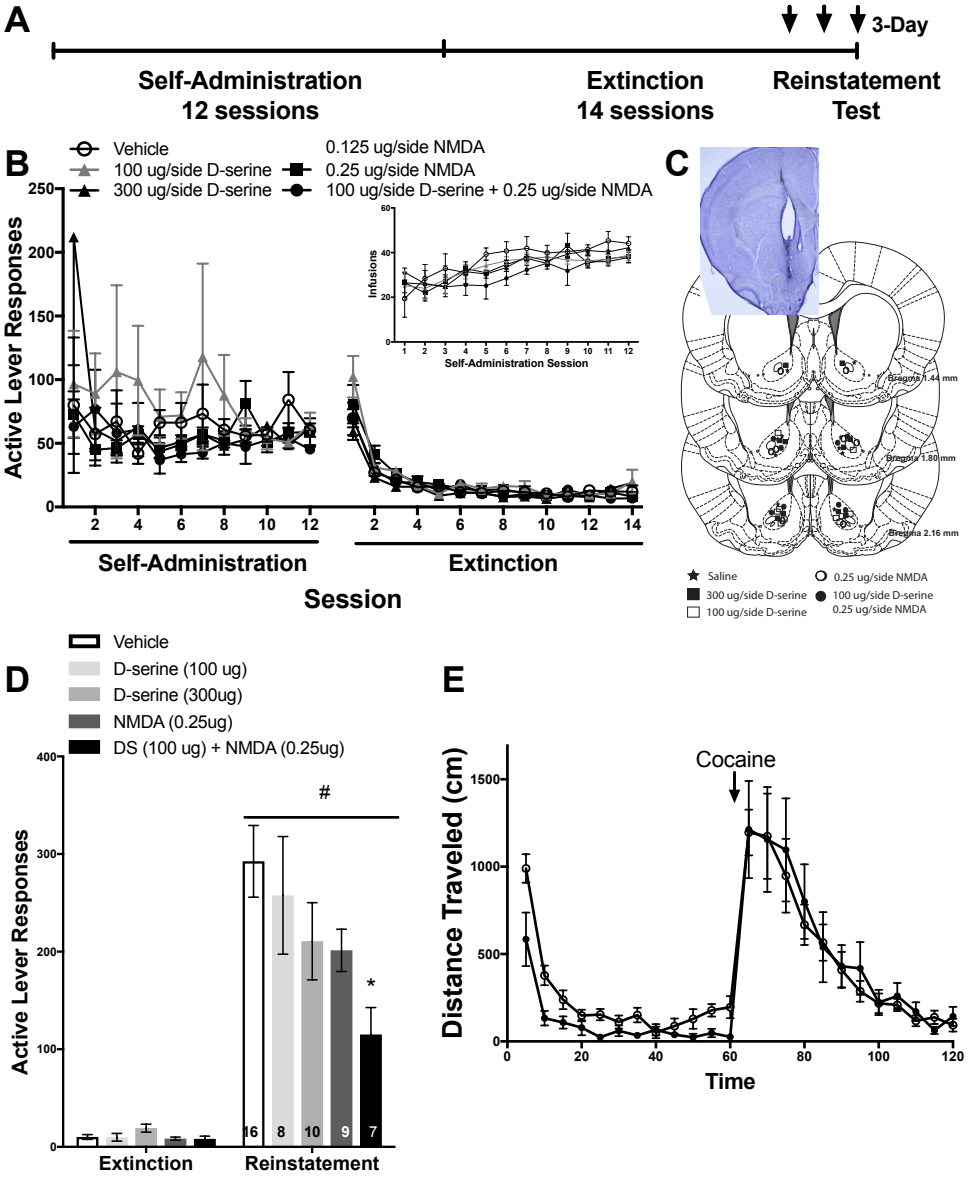


Figure 3.2. Behavioral training was similar between the groups and animals significantly reinstated cocaine seeking. (A) Timeline of study. (B) Animals readily learned to self-administer cocaine, and there were no differences in self-administration or extinction training between the treatment groups. (C) Animals who received a 1 hr reinstatement test significantly increased cocaine seeking compared to their extinction responding, however, there were no differences in reinstatement responding between the treatment groups. Hash denotes significant effect of reinstatement (#p<0.05).

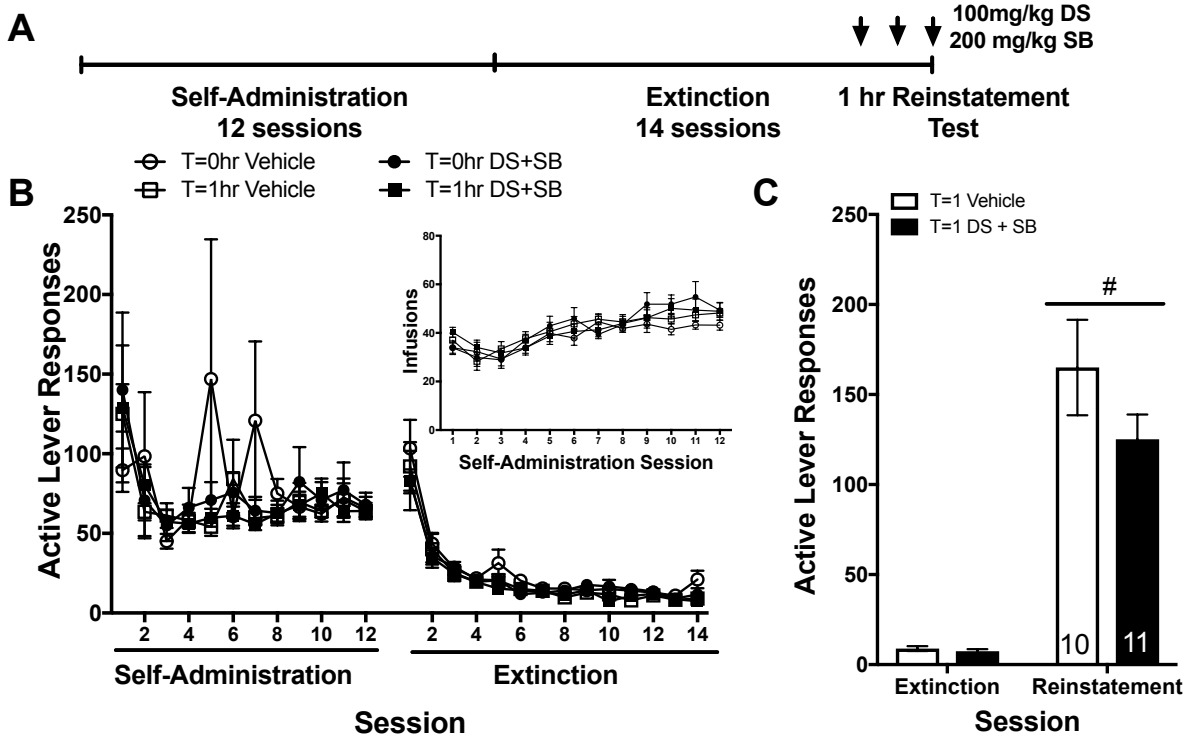


Figure 3.3 D-serine augmentation increases total protein fraction GluN1 expression in the nucleus accumbens core. Total protein fraction NMDA receptor subunits GluN1, GluN2A and GluN2B were assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-C) nucleus accumbens, (D-F) hippocampus and (G-I) striatum. Representative bands are in the order of Vehicle T=0, DS+SB T=0, Vehicle T=1 and DS+SB T=1. Asterisk denotes significant effect *vs.* vehicle T=0 (*p<0.05).

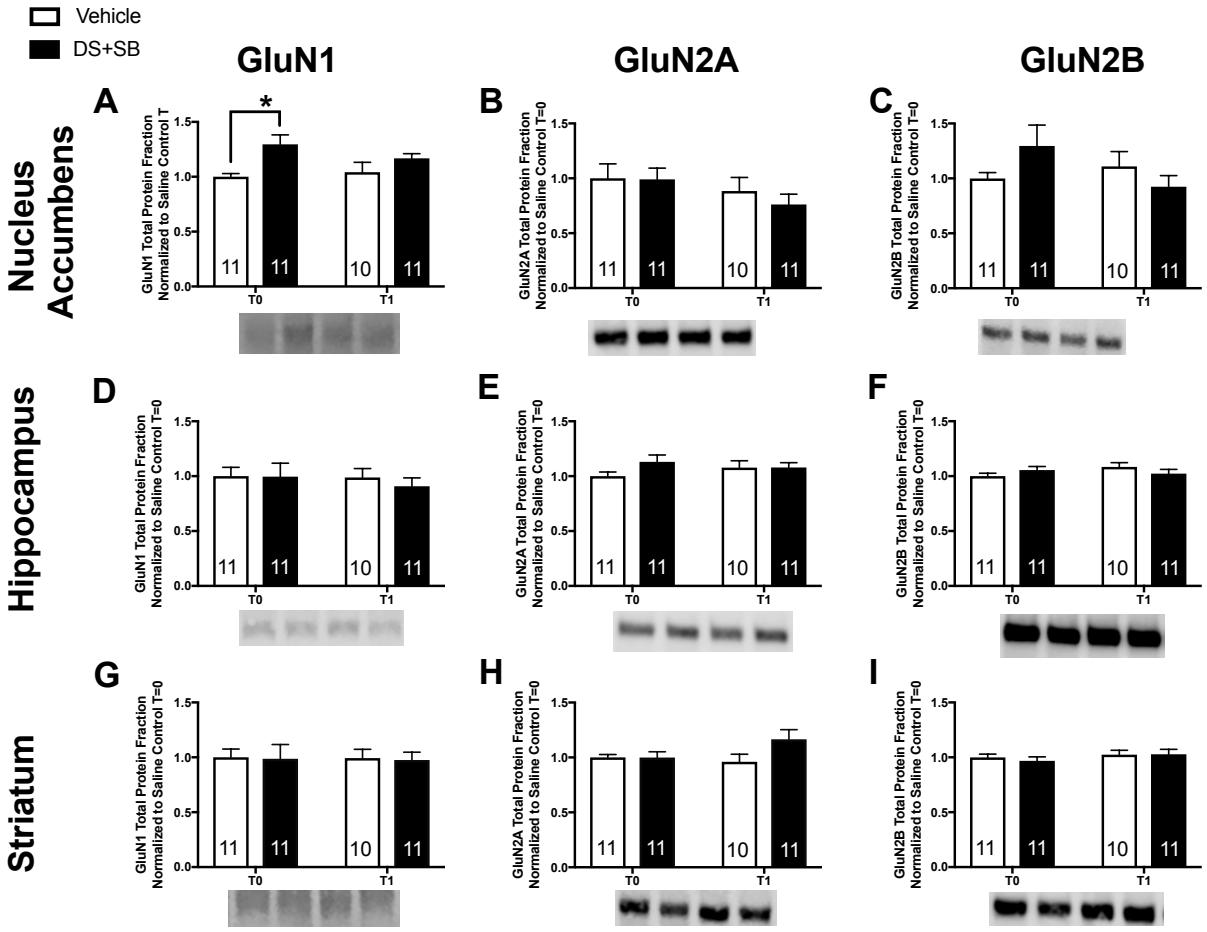


Figure 3.4. D-serine augmentation attenuates a reinstatement induced increase of total protein fraction AMPA receptor subunit GluA1 in the nucleus accumbens core. Total protein fraction AMPA receptor subunits GluA1 and GluA2 were assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-B) nucleus accumbens, (C-D) hippocampus and (E-F) striatum. Representative bands are in the order of Vehicle T=0, DS+SB T=0, Vehicle T=1 and DS+SB T=1. Asterisk denotes significant effect *vs.* vehicle T=0 (*p<0.05).

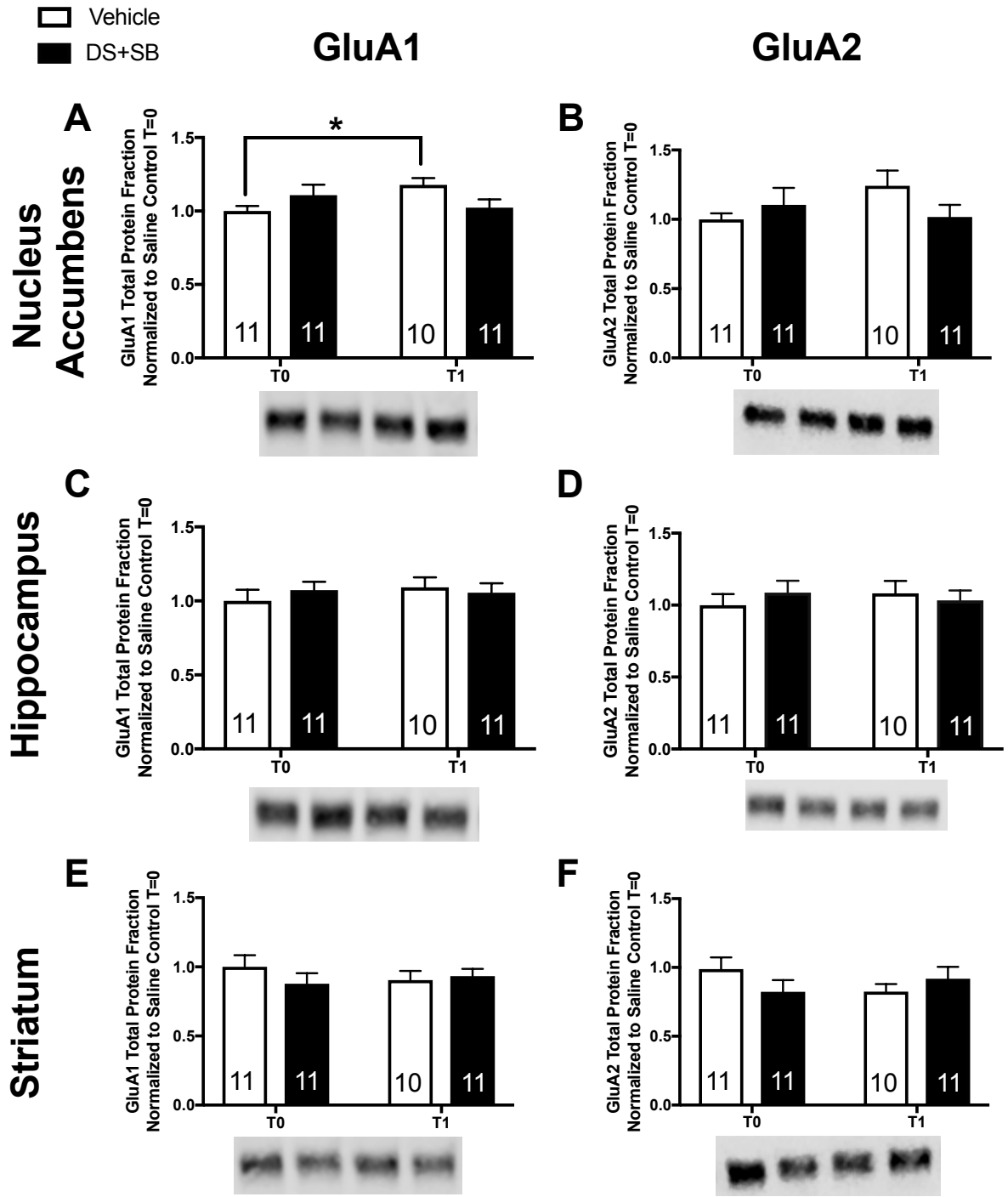


Figure 3.5 Nucleus accumbal, hippocampal and striatal surface expression of NMDA receptor subunit composition remained unchanged by D-serine augmentation.
Biotinylated fraction NMDA receptor subunits GluN1, GluN2A and GluN2B were assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-C) nucleus accumbens, (D-F) hippocampus and (G-I) striatum. Representative bands are in the order of Vehicle T=0, DS+SB T=0, Vehicle T=1 and DS+SB T=1.

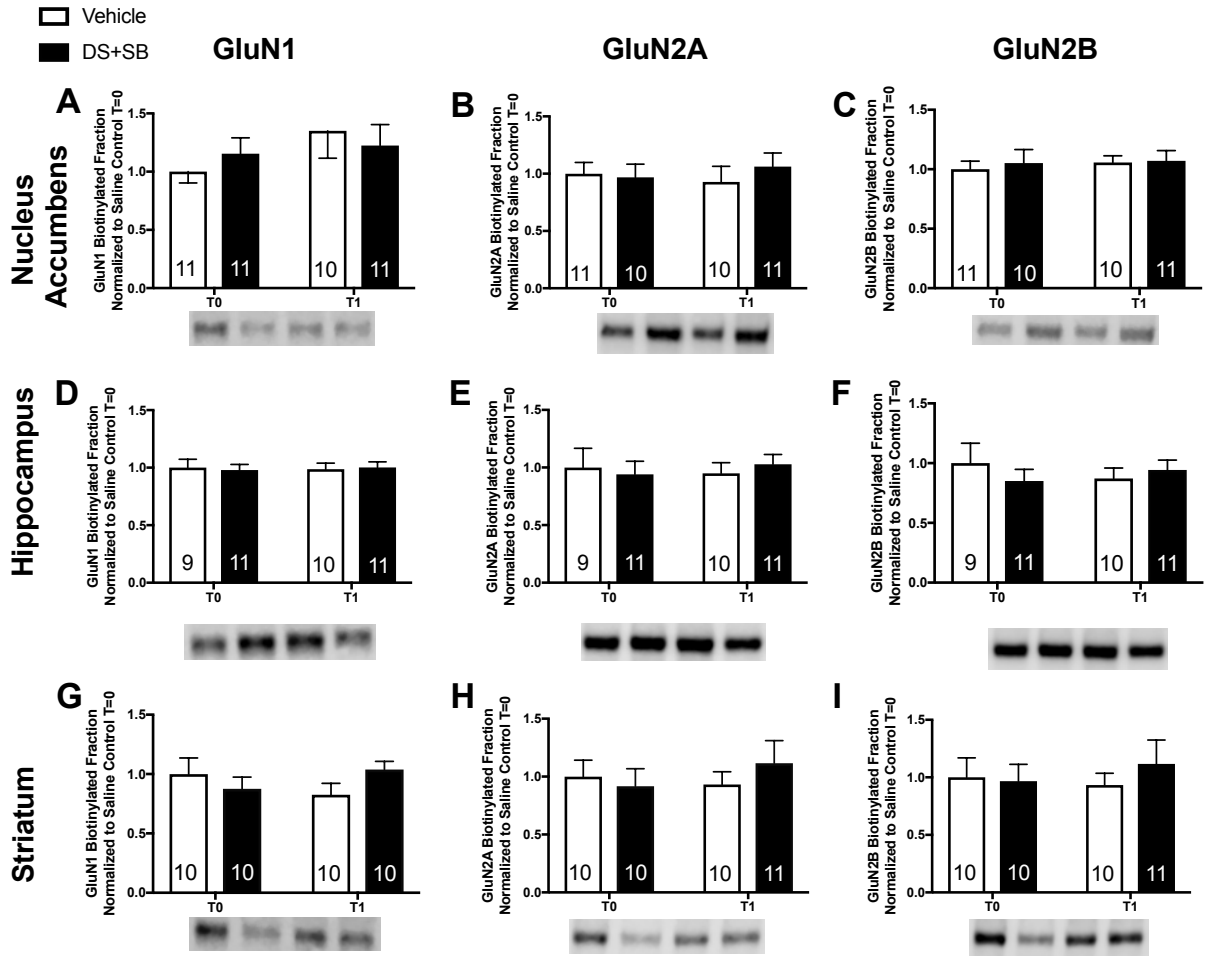


Figure 3.6 Nucleus accumbal, hippocampal and striatal surface expression of AMPA receptor subunit composition remained unchanged by D-serine augmentation. Biotinylated fraction AMPA receptor subunits GluA1 and GluA2 were assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-B) nucleus accumbens, (C-D) hippocampus and (E-F) striatum. Representative bands are in the order of Vehicle T=0, DS+SB T=0, Vehicle T=1 and DS+SB T=1.

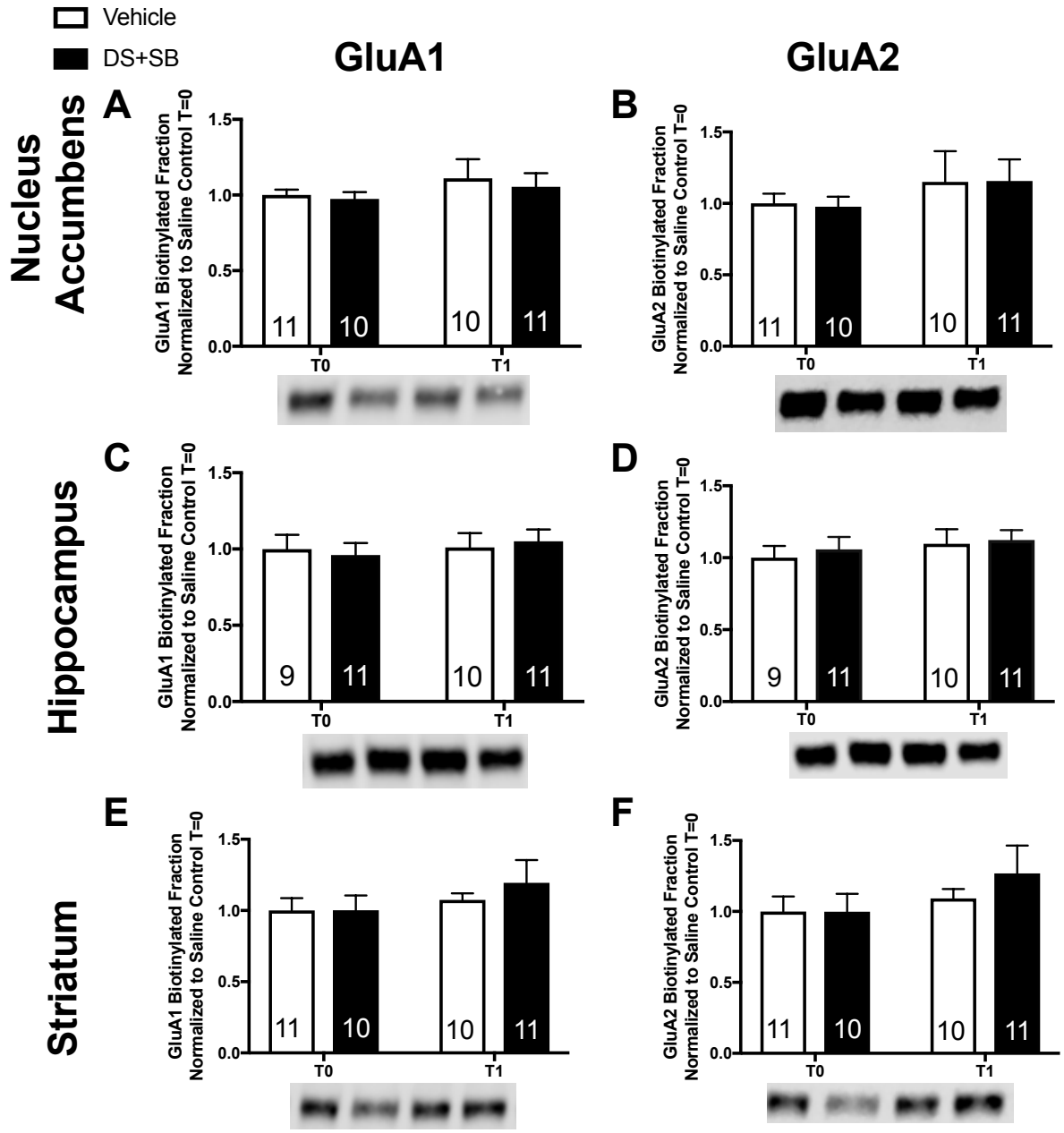


Figure 3.7. A 1 hr cocaine plus cue-primed reinstatement test increases surface expression of GLT-1 in the nucleus accumbens. Total protein and biotinylated fraction glutamate transporter (GLT-1) were assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-B) nucleus accumbens, (C-D) hippocampus and (E-F) striatum. Representative bands are in the order of Vehicle T=0, DS+SB T=0, Vehicle T=1 and DS+SB T=1. Asterisk denotes significant effect (*p<0.05).

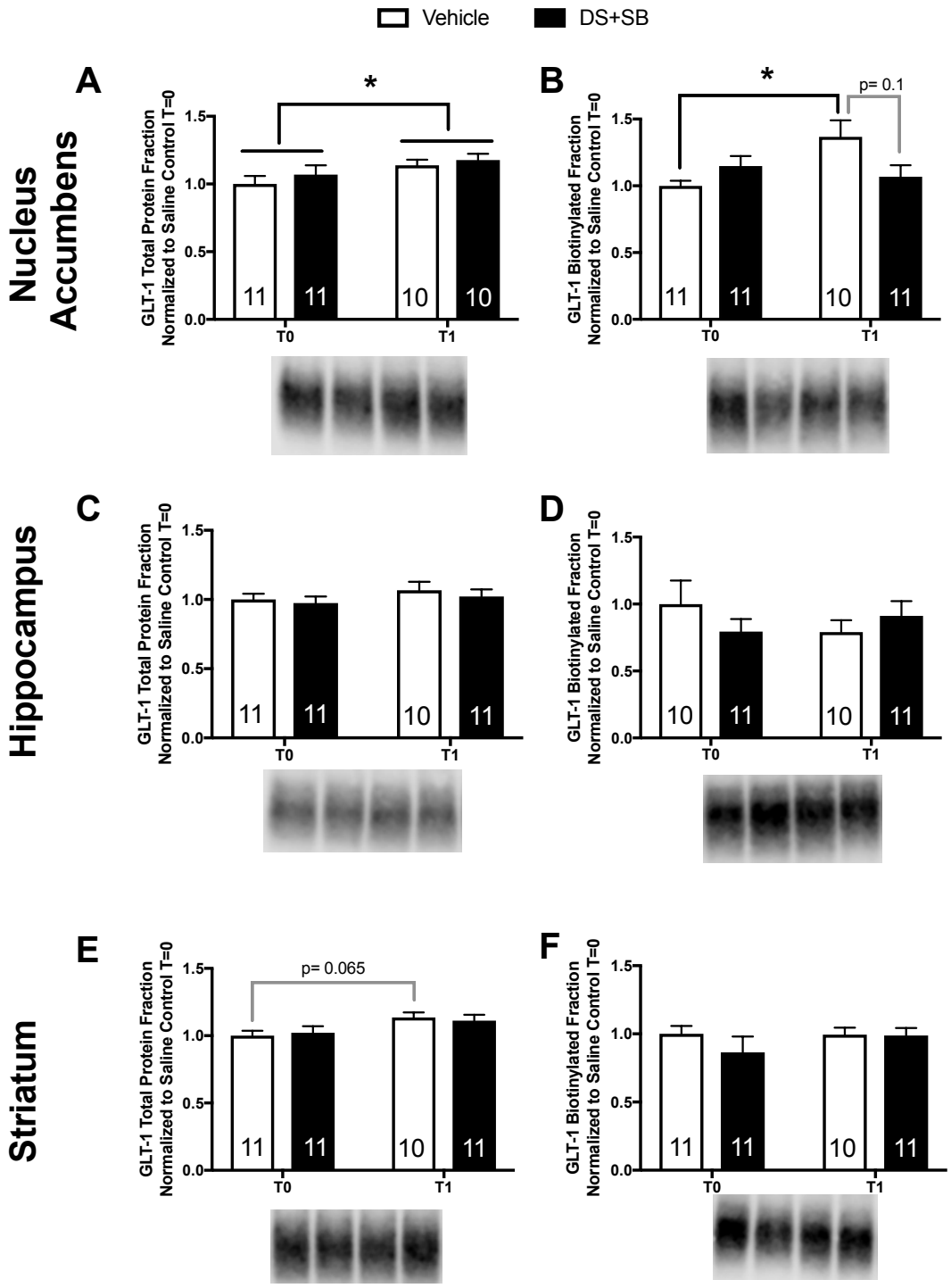
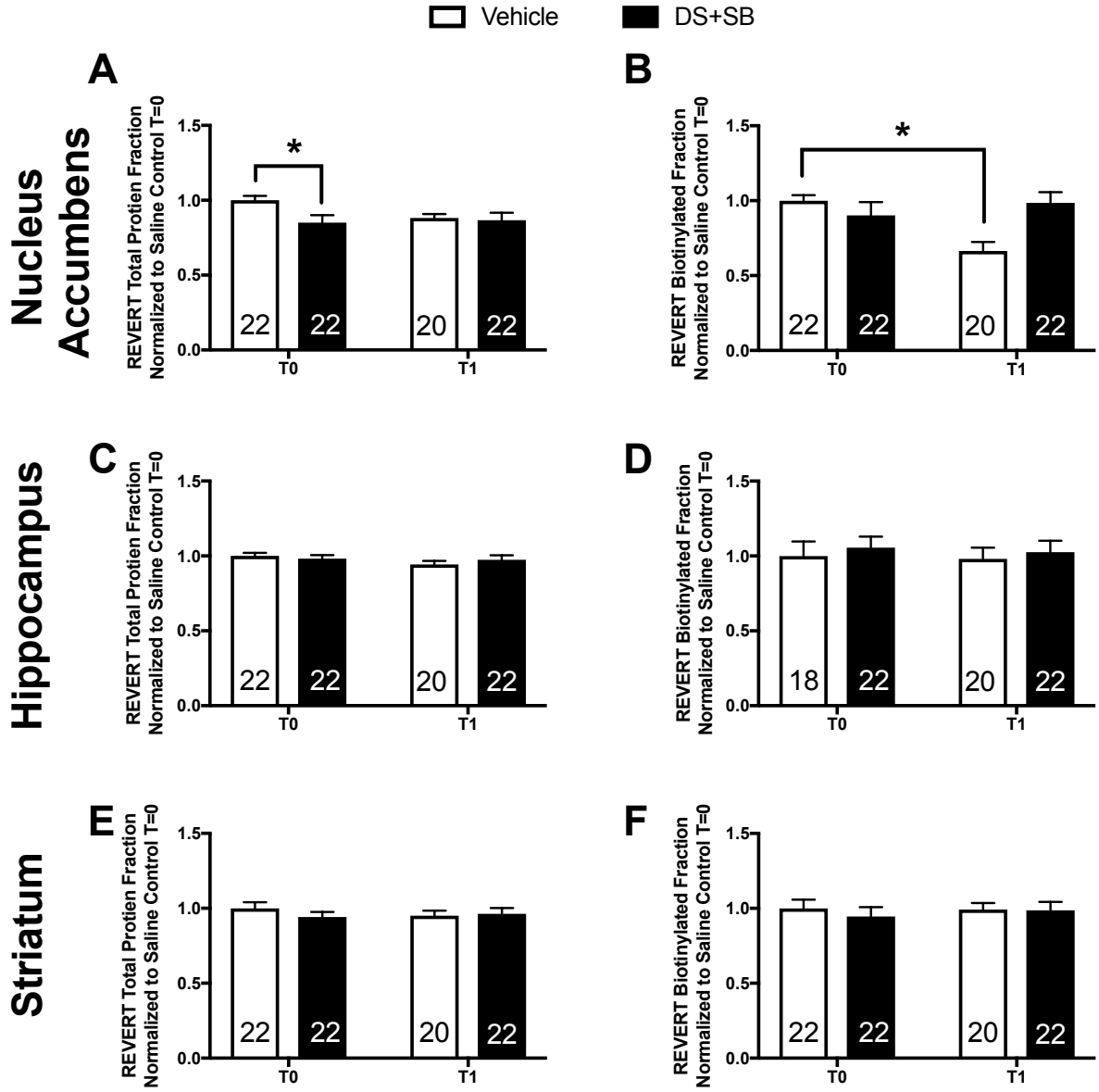


Figure 3.8 Increased surface expression of GLT-1 in the NAc core during reinstatement may be confounded by a loss of surface proteins. REVERT protein stain was significantly reduced in the NAc biotinylated fraction. REVERT protein stain quantification of the total protein fraction and biotinylated fraction between 75-100 kDa was assessed at T=0 and T=1hr in animals treated with DS (100 mg/kg) and SB (200 mg/kg) or vehicle in the (A-B) nucleus accumbens, (C-D) hippocampus and (E-F) striatum. Asterisk denotes significant effect *vs.* vehicle T=0 (*p<0.05).



CHAPTER 4:

Nucleus Accumbens Core Cellular Properties of Cocaine and D-serine Augmentation.

Introduction

Limbic brain regions such as the medial prefrontal cortex (mPFC), ventral tegmental area, hippocampus and amygdala send projections to the NAc. The projections specifically target core or shell sub regions, however they are interconnected and can process information in parallel or feedforward connections (Alexander, Crutcher, & DeLong, 1990; Groenewegen, Galis-de Graaf, & Smeets, 1999; Haber, 2003). Cocaine indirectly mediates glutamate signaling on these limbic to NAc projections, producing persistent maladaptive changes in neuronal function and plasticity, and driving cocaine seeking behaviors (Kalivas et al., 2009).

Because the pharmacologic effects of acute cocaine are mediated by blockade of reuptake of dopamine, serotonin and norepinephrine, changes in glutamatergic signaling are believed to arise as a consequence of long-term hyperdopaminergia. Withdrawal from repeated self-administration of cocaine leads to reduced basal levels of extracellular glutamate in the NAc core, and a non-contingent cocaine challenge leads to a rapid increase in glutamate release (McFarland et al., 2003). These changes are in part due to cocaine withdrawal-induced decreases in expression of the cysteine-glutamate antiporter subunit system xc-, responsible for extracellular glutamate tone, and glutamate transporter GLT-1, responsible for the reuptake of over 90% of synaptically released glutamate (Kalivas, 2009). These glutamatergic changes in the NAc core drive cocaine seeking, as reversal of these alterations in system xc- and GLT-1 attenuate reinstatement responding (Baker et al., 2003; Knackstedt et al., 2009; LaCrosse, Hill,

& Knackstedt, 2016; LaCrosse et al., 2017; Reissner et al., 2014; Reissner et al., 2015; Trantham-Davidson et al., 2012).

Further implicating glutamatergic adaptations in the NAc as drivers of cocaine seeking, glutamatergic ionotropic signaling is altered following cocaine exposure and prolonged withdrawal. NAc core synapses are potentiated following extinction from cocaine self-administration, as measured by AMPA:NMDA ratios, and become further potentiated after exposure to drug associated cues or the drug itself (Gipson et al., 2013; Shen et al., 2014). Because of the nature of the AMPA:NMDA ratio, this basal potentiation of the synapse could be, at least in part, a result of failure to activate NMDA receptors. The synaptic potentiation following cue and drug primed reinstatement is increased at 15 (cue-prime) or 45 (drug-prime) minutes into reinstatement and is returned back to basal levels in as little as 120 minutes (Gipson et al., 2013; Shen et al., 2014).

It is well established that enhanced AMPA receptor expression and activity in the NAc contributes to cocaine seeking behavior (Heath D. Schmidt & R. Christopher Pierce, 2010); however, the role of NMDA receptors is less clear. For example, there is conflicting evidence regarding the role of NMDA receptors in cocaine reinstatement. Some have reported that NMDA receptor antagonism in the NAc core or shell results in spontaneous recovery of seeking behavior (Backstrom & Hyytia, 2007) and another study reported that the same drug, ((2R)-amino-5-phosphonovaleric acid (APV), infused into the NAc core attenuates cue induced-reinstatement to cocaine seeking (Famous et al., 2008). Moreover, other studies have found no effects of NMDA receptor antagonism on reinstatement behavior (Cornish et al., 1999).

Reports regarding cocaine-induced adaptations in NMDA receptor function are also mixed. Curcio et al (2013) reported that NAc NMDA receptor activity is impaired following non-contingent cocaine exposure and 24-hr withdrawal (Curcio et al., 2013), however evoked NMDA receptor mediated currents remain unchanged after non-contingent exposure and 2 weeks withdrawal (Joffe & Grueter, 2016). Further, NMDA receptor subunit GluN1 protein is increased

following prolonged withdrawal from non-contingent cocaine exposure (Ghasemzadeh, Mueller, & Vasudevan, 2009; Scheggi et al., 2002; Schumann & Yaka, 2009) and cocaine self-administration (Lu et al., 2003), but also increases have been found in GluN2A and GluN2B (Al-Hallaq, Conrads, Veenstra, & Wenthold, 2007; Ghasemzadeh et al., 2009; Hafenbreidel et al., 2014; Loftis & Janowsky, 2002; Schumann & Yaka, 2009). It should be noted that some studies report no change in expression of GluN1 and GluN2A/B (Ferrario, Goussakov, Stutzmann, & Wolf, 2012) and even a decrease in GluN1 (Self, Choi, Simmons, Walker, & Smagula, 2004)in the NAc, clouding the picture of cocaine induced NMDA receptor adaptations.

The NAc shell has been investigated for changes in NMDA receptor signaling, but none have been found (Ferrario et al., 2012; Pavel I. Ortinski, Vassoler, Carlson, & Pierce, 2012; Thomas, Beurrier, Bonci, & Malenka, 2001). Yet, the NAc core has not been as extensively studied (Joffe & Grueter, 2016), potentially because NAc shell upregulation of CP-AMPA receptors has been well defined [for review see (Wolf, 2012)], leading to questions of NMDA receptor involvement. But also, the contradictory findings of NMDA receptors in the core (P. I. Ortinski, 2014; Heath D. Schmidt & R. Christopher Pierce, 2010) are potentially responsible for the lack of information on cocaine induced adaptations to NMDA receptor function. However, recent data from our laboratory indicate that that NMDA receptor function in the NAc core following cocaine self-administration and prolonged withdrawal should be investigated further.

We recently established that astrocytes in the NAc core are smaller, and have reduced colocalization with the synapse, potentially disrupting volume transmission of astrocyte-derived D-serine at the synapse (Scofield, Li, et al., 2016). D-serine is a co-agonist of NMDA receptors at the glycine modulatory site (GMS), and is integral for NMDA receptor function. Further, experiments in this dissertation indicate that systemic D-serine augmentation, and direct stimulation of NAc core NMDA receptors with D-serine and NMDA attenuate cocaine seeking. We then found that D-serine augmentation had no effect on the surface expression of NMDA receptors, which lead me to investigate the potential mechanism of D-serine augmentation to be

more efficiently activating NMDA receptors, rather than increasing the NMDA receptor population. Here, I characterize NMDA receptor function in the NAc core following cocaine self-administration and extinction, and investigate the effects of systemic D-serine augmentation on both NMDA receptor and synaptic function.

Methods.

Animals.

Male Sprague Dawley rats (Harlan Farms, Raleigh NC), aged approximately 6-8 weeks and weighing 260-300 grams at the time of surgery were used in these experiments. Rats were individually housed in a temperature controlled environment on a reversed 12:12 light:dark (lights off at 0700) schedule. Following a week of habituation, animals were put on a food restricted diet of 20 g/day (Envigo Teklad laboratory animal diet). All procedures were approved by the University of North Carolina's Institutional Animal Care and Use Committee.

Behavioral training.

See detailed description of training in chapter 2. Briefly, animals were trained to self-administer cocaine in 12 sessions, and then moved to extinction training for 14 sessions. Treatment of D-serine augmentation (100 mg/kg D-serine and 100 mg/kg or 200 mg/kg Sodium Benzoate) or vehicle were administered 3 hrs prior to the last two extinction training sessions and on test day, as described in previous experiments.

Electrophysiology and NMDA currents.

Rats were anesthetized with pentobarbital (65 mg/kg) and perfused transcardially with oxygenated cold modified artificial cerebral spinal fluid (aCSF) - NMDG-HEPES recovery solution and decapitated (S. Zhao et al., 2011). Coronal slices of the NAc were taken with a vibratome (220 μm; Leica VT1200S) in oxygenated ice-cold cutting solution. The NAc slices

were incubated at 32^{o}C for at least 10 min in oxygenated NMDG-HEPES recovery solution and then one hr in oxygenated modified HEPES holding ACSF solution at room temperature (S. Zhao et al., 2011). Slices were transferred to the recording chamber and neurons visualized with DIC microscopy using a 60X water-immersion objective on an upright microscope (Scientifica) perfused at 2-3 ml/min with 32^{o}C ACSF with 50 μM picrotoxin, to block $GABA_A$ currents. Whole-cell patch-clamp recording were taken from NAc core MSNs by a Multiclamp 700B amplifier using glass pipettes with a resistance of 2.5–3.5 MΩ filled with an internal solution containing (in mM): 10 Cs-Cl, 130 CsOH, 130 gluconic acid, 10 HEPES, 11 EGTA, 2 ATP, 3 GTP, and 1 $CaCl_2$. pH was adjusted to 7.3 with CsOH (300 mOsm). After establishing a whole-cell voltage-clamp recording, the resting membrane potential, the membrane resistance, membrane capacitance, and access resistance were monitored during the recording. Cells were excluded when the access resistance deviated by greater than 20% during the recording. Recordings were filtered at 4 kHz, digitized at 10 kHz via Digidata 1440A (Axon Instruments, Inc.) and saved to a computer using pCLAMP10 (Molecular Devices). To obtain AMPA:NMDA ratios, EPSCs were measured in response to approximately 1mA stimulation by a concentric bipolar electrode placed approximately 100-200 μM dorsomedial to the patched neuron. Picrotoxin (50 μM) was added to the bath to Block GABA A-mediated currents. AMPA-mediated EPSCs were recorded at -80mV and NMDA EPSCs were recorded at +40mV in the presence of CNQX (10 μM) to block AMPA receptor currents. NMDA receptor mediated currents were evoked extracellular electrical stimulation (0.10 ms) of increasing intensity (0.5-2.5 mA) with an intertrial interval of 20 s, as described previously (Hong et al., 2009).

Data analysis.

Repeated measures analysis of variance (ANOVA) was used to compare the behavioral data of the groups (active lever responding, inactive lever responding and infusions). Analysis of electrophysiology data was performed in Clampfit 10 (Axon Instruments, Union City, CA) followed by a three-way analysis of variance (self-administered drug x treatment x current) to compare the groups. Sidak's multiple comparison *post hoc* corrections were used to compare groups.

Results.

Behavioral training.

There were no differences between groups during cocaine self-administration or extinction (comparing cocaine-administering only; Table 4.1 and Figure 4.1B) or saline self-administration or extinction (comparing saline-administering only; Table 4.1 and Figure 4.1B). All cocaine self-administering rats reliably extinguished lever responding during extinction training (Sidak's multiple comparisons test, $p<0.05$).

Cocaine self-administration and prolonged withdrawal potentiate NAc core MSN and D-serine augmentation rescues this effect.

Following cocaine self-administration and extinction, MSNs exhibit potentiated NAc core synaptic strength compared to cocaine naïve animals (Gipson et al., 2013; Pavel I. Ortinski et al., 2012; Shen et al., 2014). We replicated this effect (significant interaction effect $F_{(1,40)}=14.1$, $p<0.01$, *Sidak's* test of multiple comparison, Saline/Veh > Cocaine/Veh; Figure 4.1C). Further, D-serine augmentation attenuated this potentiation of the synapse as cocaine self-administering animals with D-serine augmentation showed significantly lower AMPA:NMDA ratio than cocaine self-administering vehicle animals (*Sidak's* test of multiple comparison, $p<0.05$; Figure 4.1C), however they were no different than saline self-administering vehicle animals ($p=0.83$). Further,

there was a non-significant trend for D-serine augmentation in saline self-administering animals' AMPA:NMDA ratio to be greater than D-serine augmented cocaine self-administering animals (p=0.07), although D-serine augmented saline self-administering animals were no different than saline self-administering vehicle animals (p=0.41).

D-serine augmentation increases evoked NMDA receptor currents.

A three-way mixed ANOVA with current as the repeated measures factor, and group (cocaine vs. saline self-administering rats) and treatment (vehicle vs. D-serine augmentation) as between subject factors, was run to examine differences in evoked NMDA receptor currents over increasing stimulation intensity. There was a non-significant trend for a three-way interaction between group, treatment and stimulation ($F_{(1,29)}$=2.1, p=0.07). There was a significant effect of stimulation ($F_{(1,29)}$ = 96.3, p <0.01), where at increasing stimulations the evoked NMDA currents increased (*Sidak's* multiple corrections test, p<0.05; Figure 4.1D). There was a significant stimulation x treatment interaction ($F_{(1,29)}$ = 15.8, p <0.01), and *post hoc* analysis revealed that NMDA receptor currents in MSNs from D-serine-augmented rats were greater than vehicle animals at all stimulation intensities (*Sidak's* multiple corrections test, p<0.05; Figure 4.1D). There were no differences in saline self-administering and cocaine self-administering evoked NMDA receptor currents as the interaction between stimulation and group was not significant ($F_{(1,29)}$ = 0.2, p=0.97). These results indicate that treatment with D-serine augmentation, regardless of self-administration of cocaine or saline, increased evoked NMDA receptor currents compared to vehicle control animals.

Discussion.

Cocaine self-administration and prolonged withdrawal has been shown to change excitatory neuroplasticity in NAc core MSN, such as increased strength of AMPA relative to NMDA-mediated currents (AMPA:NMDA ratio) compared to cocaine naïve controls (Gipson et

al., 2013; Shen et al., 2014). Here, we replicated this effect showing that extinguished cocaine self-administering animals had significantly greater AMPA:NMDA ratios than cocaine-naïve control animals. Additionally, we probed the ability of systemic D-serine augmentation to attenuated this increase in synaptic potentiation. D-serine augmentation rescues these cocaine-induced synaptic alterations in NAc core MSN synapses. Lastly, D-serine augmentation increased evoked NMDA receptor mediated currents in both saline- and cocaine-extinguished rats, indicating that NAc core NMDA receptors may contribute to the restored AMPA:NMDA ratio in treated animals with a cocaine history. Collectively, these results add further evidence that increasing NMDA receptor activation in the NAc core is potentially the driving force behind the behavioral effects of D-serine augmentation.

Association between NAc core synaptic potentiation and established D-serine augmentation effect

As presented above (Chapter 2 and 3), both systemic D-serine augmentation and intra-NAc activation of NMDA receptors attenuate cocaine plus cue-primed reinstatement to cocaine seeking. Now, we have shown that NAc core MSN AMPA:NMDA ratios are increased in cocaine extinguished animals and that our treatment of D-serine augmentation attenuates this effect. These findings further support the role of NAc core MSN synaptic changes in driving cocaine seeking behaviors, but also indicate that these maladaptive changes are dynamic and can be altered with treatment.

It was beyond the scope of this work to investigate synaptic changes during the reinstatement test, however, it would do well to investigate the effects of D-serine augmentation on known reinstatement-dependent transient changes in NAc core MSN synaptic potentiation (Gipson et al., 2013; Shen et al., 2014). It's possible that D-serine augmentation also reverses the rapid synaptic plasticity induced by cues associated with cocaine (Gipson et al., 2013) and cocaine itself (Shen et al., 2014). Further, this transient synaptic potentiation has not been

investigated following a cocaine plus cue-primed reinstatement test. Cocaine associated cues and an acute cocaine dose have differing timelines for initiation of synaptic potentiation (Gipson et al., 2013; Shen et al., 2014), and so it would be necessary to determine the timeline of a combined cocaine plus cue prime transient synaptic potentiation, in addition to the effects of D-serine augmentation on synaptic potentiation.

Furthermore, this transient, LTP-like increase in AMPA:NMDA ratio is likely a result of increased AMPA receptor signaling (Shen et al., 2014), and strengthening NMDA receptor signaling can counteract these effects. However, the facilitation of AMPA receptor signaling is not dependent on rapid changes in AMPA receptor surface expression or subunit expression [Chapter 4;(Shen et al., 2014)], as previously thought. In Chapter 4 of this dissertation we see no change in AMPA receptor subunit composition between vehicle treated animals at the two time-points: basally and 1 hr into a cocaine plus cue-primed reinstatement test. These results negate a possibility that elevated AMPA receptor expression increases AMPA receptor currents, thereby potentiating the synapse as measured by AMPA:NMDA ratios. It's possible that despite no change in protein concentration, AMPA receptor currents are increased in the NAc core due to increased sensitivity of AMPA receptors to activate or increased decay times that would allow for greater current in the same number of receptors. Interestingly, in the rat hippocampus, D-serine administration inhibits AMPA receptor mediated currents, and may be a competitive antagonist of AMPA receptors (Gong, Zabek, & Bai, 2007). These possibilities warrant further investigation.

D-serine augmentation increased evoked NMDA receptor mediated currents

Interestingly, we found no difference in evoked NMDA receptor mediated currents between cocaine and saline self-administering animals, as we had expected. These results are congruent with previous findings, where no differences were found in electrically evoked NMDA receptor properties or NMDA:AMPA ratio after non-contingent cocaine and 2 weeks of

abstinence compared to cocaine naïve animals (Joffe & Grueter, 2016) and also no change was found in AMPA:NMDA ratio 24hr after non-contingent cocaine (Pavel I. Ortinski et al., 2012). Collectively, these findings indicate despite a cocaine withdrawal induced increase in NMDA receptor protein (P. I. Ortinski, 2014), it has no effect on the evoked NMDA receptor mediated currents in the NAc core. Our original hypothesis that extinguished cocaine self-administering animals have a deficit in D-serine at the synapse, due to reduced astrocytic contact with the synapse (Scofield, Li, et al., 2016), is still applicable. It is possible that NMDA receptors are present, and can be evoked *in vivo* electrophysiology recordings, but are not endogenously stimulated due to insufficient co-agonist affecting behavioral output. Interestingly, these results also point to AMPA receptors as driving the differential changes in AMPA:NMDA ratio between saline self-administering and cocaine self-administering D-serine augmented animals.

It's important to note that increased surface expression and exchanging of calcium impermeable AMPA receptors for calcium permeable AMPA receptors have been reported extensively in the NAc after long-access and incubation model of cocaine craving, but there is limited research on short access cocaine self-administration and extinction induced AMPA receptor alterations [for a review see (Wolf & Tseng, 2012)]. What is known, is that short access cocaine self-administration and prolonged withdrawal upregulates calcium impermeable but not calcium permeable AMPA receptors (McCutcheon, Wang, Tseng, Wolf, & Marinelli, 2011; Purgianto et al., 2013), indicating multiple levels of adaptations to the glutamatergic system that are dependent on cocaine exposure and length of cocaine withdrawal. It is noteworthy, that the experiments reporting changes in AMPA receptor rectification, and thus a change of subunits expressed at the surface, were observed in NAc core MSNs of cocaine self-administering abstinent animals (Conrad et al., 2008; McCutcheon et al., 2011), and extinction training may eliminate these changes as there are reports of unaltered subunit composition in extinguished cocaine self-administering animals (Shen et al., 2014).

Interestingly, D-serine augmentation increased evoked NMDA mediated receptor currents in both cocaine and saline self-administering animals, but only cocaine self-administering animals demonstrated a decrease in NAc core AMPA:NMDA ratio. In fact, saline self-administering D-serine augmented animals had a trend of a greater AMPA:NMDA ratio than cocaine self-administering D-serine augmented animals. These effects are reminiscent of the findings Curcio and colleagues (2013) where D-serine added to the wash solution increased NMDA receptor mediated field potentials to a greater extent in non-contingent cocaine exposed animals than in cocaine naïve controls (Curcio et al., 2013). This was taken to indicate hypofunctioning NMDA receptors in the NAc core following cocaine experience. Our results and others (Joffe & Grueter, 2016) indicate that NMDA receptors are capable of functioning as well as their cocaine naïve control animals, giving evidence that changes in AMPA receptor signaling contribute to the changes in AMPA:NMDA ratio seen between saline self-administering and cocaine self-administering animals. Alternatively, it is also possible that the conditions set in our slice recordings are different than the endogenous conditions, and could be affecting our results. Further, our results are an indication that after D-serine augmentation NAc core NMDA receptors are more sensitive to activation, even when D-serine is not in the recording solution.

Conclusions

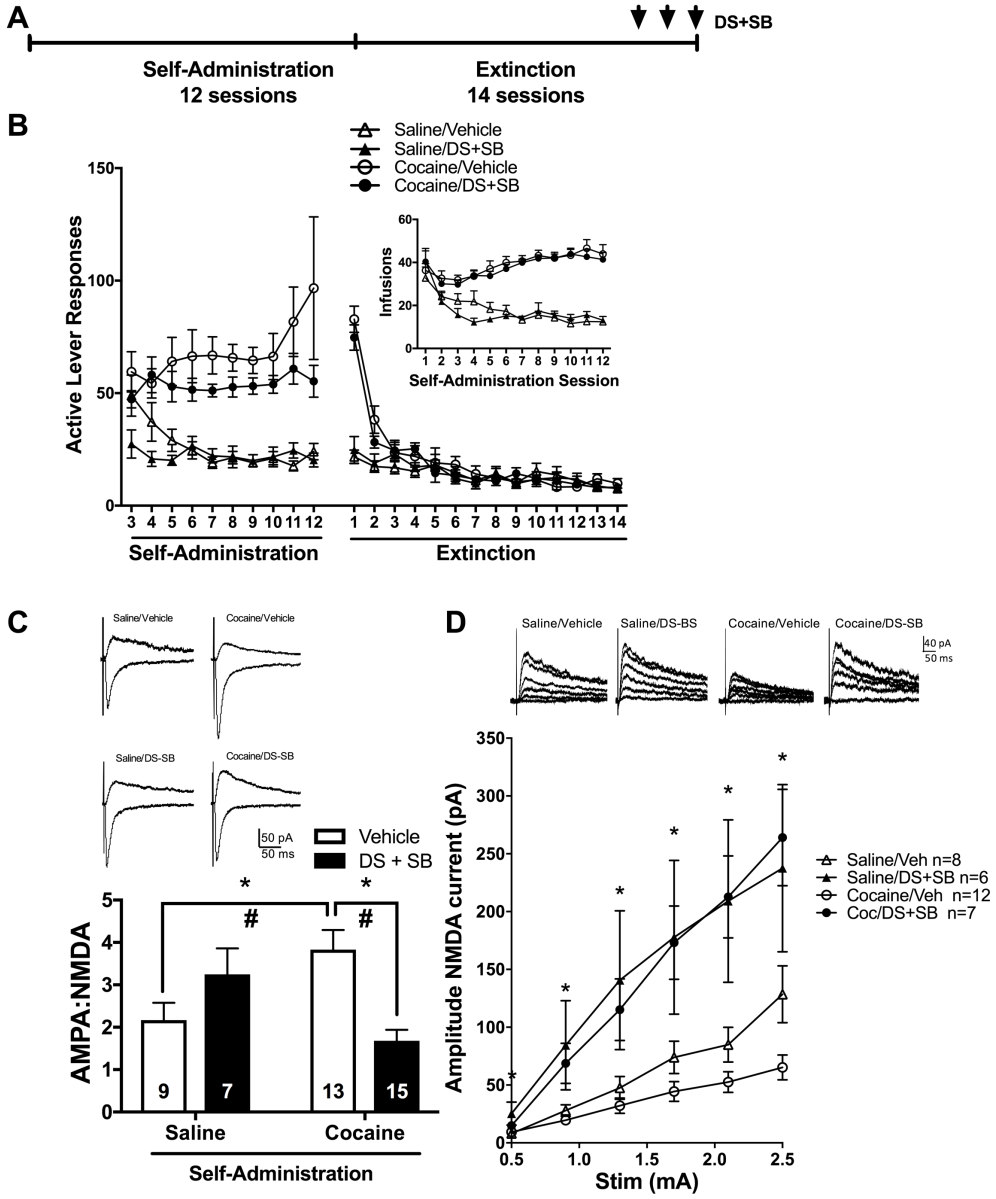
These findings add to a growing evidence that following cocaine self-administration and extinction training NAc core MSN are potentiated compared to naïve control animals and that depotentiating the synapse can inhibit cocaine seeking behaviors (Gipson et al., 2013; Shen et al., 2014). We add further evidence elucidating of the complex role of NMDA receptors in cocaine reinstatement behaviors, an area of research that is murky at best (P. I. Ortinski, 2014; Heath D. Schmidt & R. Christopher Pierce, 2010). 3 day D-serine augmentation increases evoked NMDA receptor currents regardless of self-administration history, however only treatment in cocaine self-administering animals depotentiated the synapse, indicating a role for

AMPA receptors in the effect. Collectively, these experiments highlight that investigation into the cellular effects of reinstatement behaviors is a promising area of study for the development of pharmacotherapeutics aimed at reducing relapse.

Table 4.1. Behavioral Data.

Self-Administration	Behavior	F (interaction)	P	F (Session)	P	F(Treatment)	P
Saline	Self-Ad Active Lever	F (9, 180) = 2.109	P=0.0309	F (9, 180) = 3.174	P=0.0014	F (1, 20) = 0.8716	P=0.3617
	Extinction Active Lever	F (13, 260) = 0.3332	P=0.9864	F (13, 260) = 4.828	P<0.0001	F (1, 20) = 0.006221	P=0.9379
	Infusions	F (11, 198) = 1.181	P=0.3020	F (11, 198) = 8.682	P<0.0001	F (1, 198) = 0.1954	P=0.6590
Cocaine	Self-Ad Active Lever	F (9, 243) = 0.7431	P=0.6692	F (9, 243) = 1.141	P=0.3344	F (1, 27) = 2.134	P=0.1556
	Extinction Active Lever	F (13, 338) = 1.451	P=0.1345	F (13, 338) = 101.3	P<0.0001	F (1, 26) = 0.6167	P=0.4394
	Infusions	F (9, 225) = 0.3053	P=0.9726	F (9, 225) = 10.46	P<0.0001	F (1, 25) = 0.2521	P=0.6200

Figure 4.1. D-serine augmentation attenuates cocaine induced synaptic potentiation and increases evoked NMDA receptor currents. (A) Study timeline, systemic treatment was given on the last 2 days of extinction training (100 mg/kg D-serine and 100 mg/kg SB) and on electrophysiology test day (100 mg/kg D-serine and 200 mg/kg SB). (B) Self-administration and extinction training were similar between the treatment groups. (C) AMPA:NMDA ratio and (D) evoked NMDA receptor mediated currents in NAc core MSN with example traces. Hash denotes significant effect *vs.* vehicle T=1hr. Asterisk denotes significant effect of D-serine augmentation *vs.* vehicle treatment (*p<0.05).



CHAPTER 5

General Discussion.

D-serine's role as an astrocyte-derived co-agonist of NMDA receptors make it a novel target for psychostimulant abuse disorders, which are characterized by alterations in NAc core glutamate homeostasis (Kalivas, 2004; Scofield, Heinsbroek, et al., 2016; Scofield & Kalivas, 2014) and recently, astrocyte morphological changes (Scofield, Li, et al., 2016). Studies thus far investigating the effects of D-serine administration on psychostimulant exposure have mainly focused on strengthening learning and memory of extinction training (Hafenbreidel et al., 2014; Hammond et al., 2013; Hammond & Wagner, 2013; Kelamangalath et al., 2009; Kelamangalath & Wagner, 2010; Z. Q. Liu et al., 2016) or cocaine sensitization (Curcio et al., 2013; Fernandez-Espejo, Ramiro-Fuentes, Portavella, & Moreno-Paublete, 2008; Z. Q. Liu et al., 2016; Puhl et al., 2015; Yang et al., 2013). In these dissertation experiments, I administered D-serine augmentation at the prolonged withdrawal time point of two weeks after cocaine self-administration to allow for the investigation of NMDA receptor stimulation in cocaine reinstatement mechanisms and behavior.

As stated above, we have recently reported that astrocytes are smaller and make less contact with the synapse following self-administration of cocaine and extinction training (Scofield, Li, et al., 2016). Reduced astrocytic contact with NAc core MSN synapses could lead to reductions in volume transmission and thus reduced availability of astrocyte-derived neurotransmitters and neurotrophic factors, like D-serine, at the synapse. Hence, cocaine-induced adaptations could indirectly be reducing NMDA receptor activation because of a lack of D-serine at the synapse, although glutamate is present. This hypothesis lead to the preclinical

investigation of D-serine augmentation as potential therapeutic for combating relapse to cocaine seeking. The use of a combined cocaine plus cue-primed reinstatement test in these experiments allowed for a high degree of translational validity, as relapse to drug seeking in humans includes taking of the abused drug. Additionally, the combined cocaine plus cue-primed reinstatement test is hypothesized to induce a robust release of glutamate into the NAc core (Parsegian & See, 2014), to which reduced levels of D-serine would not be adequate to activate NMDA receptors and ultimately leading to a decrease in NMDA receptor mediated currents.

Thus, the overarching goal of this dissertation was to investigate the ability of D-serine augmentation to attenuate cocaine seeking following a cocaine plus cue-primed reinstatement event and then to characterize the mechanism by which D-serine augmentation attenuated this cocaine seeking. Using the self-administration and extinction model of cocaine abuse, findings from the present series of experiments found that systemic and intra-accumbal D-serine augmentation attenuated cocaine seeking, and that systemic D-serine augmentation depotentiated the synapse and enhanced NMDA receptor activation in the NAc core. Interestingly, the experiments presented here conclude that there are no changes in surface protein expression of glutamatergic ionotropic subunits from the NMDA and AMPA receptors following D-serine augmentation. Therefore, the experiments were unable to determine the direct mechanism of action by which D-serine augmentation is reducing relapse to cocaine seeking. However, it can be concluded that NMDA receptor induced internalization of AMPA receptors is not likely the mechanism of the D-serine augmentation effect. Together, these data enhance the current understanding of NMDA receptor involvement in cocaine relapse behaviors, such that NMDA receptors oppose reinstatement of cocaine seeking. We also provide a detailed characterization of NMDA and AMPA receptor expression and function after enhancing the NMDA receptor co-agonist D-serine.

Summary of experimental findings.

Systemic D-serine augmentation significantly attenuated cocaine seeking during a cocaine plus cue-primed test of reinstatement. Three days of D-serine augmentation, a combination of D-serine and an inhibitor of D-amino Acid Oxidase (DAO), were necessary to produce the attenuation in cocaine seeking, as acute administration had no effect on behavior. Additionally, 3-day administration of D-serine, or the DAO inhibitor sodium benzoate (SB) alone, or a low dose of SB in combination with D-serine were no different than control animal responding during reinstatement test. Further, this effect was not due to enhancing extinction training, as animals who underwent forced abstinence instead of extinction training, with 3-days of D-serine augmentation, showed attenuated cocaine seeking in cocaine plus cue-primed extinction test. These animals showed no effect of D-serine augmentation on their locomotor activity, both habituating to a novel environment and after an acute cocaine dose. Lastly, HPLC analysis of systemically treated animals indicated that only 3-days of D-serine augmentation, and not acute treatment, significantly raised D-serine levels in the nucleus accumbens core compared to vehicle treated animals. These results collectively indicate that systemic D-serine augmentation attenuated cocaine seeking by increasing D-serine levels to act upon NMDA receptors, and that the nucleus accumbens core may be the locus of this effect. Therefore, the next chapter sought to elucidate if the NAc was sufficient for this behavioral effect, and by what mechanisms might D-serine augmentation be attenuating cocaine seeking.

To investigate if the NAc core was the locus of the behavioral effects of D-serine augmentation, we directly administered D-serine and NMDA to the NAc core. Because SB caused tissue damage when injected directly (data not shown), this treatment was not used in the intra-NAc core experiments. In order to stimulate NMDA receptors in the core, we gave D-serine and NMDA together at doses that did not significantly affect seeking behavior when administered alone. D-serine plus NMDA significantly attenuated cocaine seeking when injected into the NAc core. D-serine administered alone or at a 3x higher dose had no effect on cocaine

seeking, and NMDA alone had no effect on behavior. These results cement the importance of NMDA receptors in NAc core attenuation of cocaine seeking in a cocaine plus cue primed reinstatement test, and present evidence that NAc core NMDA receptors oppose cocaine seeking following a cocaine plus cue-primed reinstatement test.

Within the NAc core, striatum, and hippocampus we found no difference in expression of AMPA subunits (GluA1 and GluA2) and NMDA receptor subunits (GluN2A and GluN2B) between systemic D-serine augmented and vehicle treated animals, implying that D-serine augmentation had no effect on transcription or translation of these receptor subunits. D-serine augmentation did increase GluN1 subunit expression in the whole homogenate compared to control animals, however this did not carry through to an increase in GluN1 surface expression. Likewise, there was no effect of D-serine augmentation on the other AMPA or NMDA receptor subunit trafficking, as there were no differences in AMPA and NMDA receptors expressed at the surface in any of the brain areas investigated. These data infer that NMDA receptor mediated AMPA internalization is unlikely to be the mechanism by which D-serine augmentation is driving behavior in the NAc, striatum or hippocampus. Further investigation is necessary to determine if these assumptions are correct and that a false negative result was not acquired due to insufficient sensitivity of the measures used to assess protein content. A change of NMDA receptor function due to decreased D-serine tone would be independent of NMDA receptor subunit expression, and so in the next chapter we sought to characterize NMDA receptor function and synaptic plasticity in the NAc core.

We assessed AMPA:NMDA ratios as a measure of synaptic potentiation. Medium spiny neurons from cocaine self-administering and extinguished animals exhibited significantly greater AMPA:NMDA ratio compared to cocaine naïve animals, as established previously (Gipson et al., 2013; Shen et al., 2014). Interestingly, D-serine augmentation in cocaine-trained animals inhibited the increase in AMPA:NMDA ratio, indicating that treatment depotentiated NAc core MSN synapses. It has been previously shown that depotentiating the synapse attenuates

reinstatement to cocaine seeking (Gipson & Kalivas, 2014; Shen et al., 2014), giving evidence that this depotentiation could be driving the previously established systemic D-serine augmentation attenuation of cocaine seeking. There was no effect of D-serine augmentation on AMPA:NMDA ratios in saline self-administering animals; although, administration of D-serine augmentation in both cocaine and saline self-administering animals increased evoked NMDA receptor current. Of note, we found no evidence that NAc core NDMA receptors are hypofunctioning as was found previously directly after non-contingent cocaine exposure (Curcio et al., 2013). However, this follows other literature finding cellular cocaine-induced changes are ameliorated following extinction training (Shen et al., 2014).

Collectively, the data from this dissertation begin to characterize that NAc core NMDA receptor activation opposes cocaine seeking behaviors and cocaine induced synaptic adaptations.

NMDA receptor makeup and function following cocaine self-administration and prolonged withdrawal.

NMDA receptor involvement in cocaine-induced adaptations is murky at best. It is generally accepted that following cocaine self-administration and extinction there is an increase in NAc and hippocampal GluN1 [(Hemby et al., 2005; Lu et al., 2003; Pomierny-Chamiolo et al., 2015); for a review see (P. I. Ortinski, 2014; H. D. Schmidt & R. C. Pierce, 2010)], although several experiments have also found no change in GluN1 subunits in these regions (Ben-Shahar et al., 2007; Ferrario et al., 2012). Additionally, there is no evidence of striatal changes in NMDA receptor expression (Ben-Shahar et al., 2007; Pomierny-Chamiolo et al., 2015). GluN1 is an obligatory subunit in the NMDA receptor complex, and as such an increase in GluN1 expression could be indicative of an overall increase in NMDA receptors following cocaine self-administration and prolonged withdrawal.

NAc core NMDA receptor involvement in cocaine-induced adaptations.

The experiments in this dissertation aid in a further understanding of NMDA receptors involvement in cocaine relapse behaviors, however, the picture still remains unclear. In departure from the non-contingent literature, we found that cocaine self-administering animals did not have hypofunctional NMDA receptors in the NAc core (Curcio et al., 2013), as there was no difference in evoked NMDA receptor mediated currents between cocaine and saline self-administering animals. However, these seemingly divergent findings maybe a result of methodology, as we did not have D-serine in our wash solution and Curcio et al. (2013) did, we also performed whole-cell patch clamp where as they recorded field potentials. Further, our findings are supported by a recent report that following non-contingent cocaine exposure and 2 weeks abstinence there were no differences in the NAc core of NMDA receptor mediated currents (Joffe & Grueter, 2016). These results indicate that if there is a cocaine withdrawal induced increase in NMDA receptor protein, as shown previously, it has no effect on the evoked current of NMDA receptors in the NAc core in slices. It is possible, that NMDA receptors are more sensitive to D-serine following cocaine self-administration and withdrawal, and we were unable to make this distinction with our recording methodology.

The conditions of slice electrophysiology recording can be very different than the conditions within the brain, and so it's possible that we were unable to capture an impairment in NMDA receptor function by using the method of evoked NMDA receptor mediated current in brain slice. For instance, in the electrophysiology experiments presented here, there was no D-serine in the wash solution when recordings were taken which could explain our lack of results. It is possible, that in extinguished cocaine-trained animals, NMDA receptor protein is present but a lack of D-serine at the synapse decreases their activation, thus adding exogenous D-serine activates these receptors. In this case, we would not see a difference in D-serine treated saline self-administering and cocaine self-administering animals in our slice recordings.

Following cocaine self-administration and extinction NAc core MSN synapses are potentiated, and D-serine augmentation attenuates this synaptic potentiation so that AMPA:NMDA ratios are no different than saline self-administering controls at basal levels. This decrease in AMPA:NMDA ratio could be a result of increased evoked NMDA receptor current, although we did not see a reduction in synaptic potentiation of saline self-administering D-serine augmented animals, indicating that this decrease in synaptic potentiation is selective to cocaine experienced animals. It's important to note that the measure of synaptic potentiation is a ratio, and as such NMDA receptor currents may not be the only factor contributing to this change in potentiation. AMPA receptor mediated currents could also be affected by D-serine augmentation, in a protein expression independent manner, which we did not explicitly experimentally address, and warrants further investigation.

NAc core reinstatement-induced synaptic adaptations

Previous studies have shown that the AMPA:NMDA ratio is increased following cocaine self-administration (Shen et al. 2014, Gipson et al. 2013) and that during a reinstatement event NAc core synapses are further potentiated (Gipson et al., 2013; Shen et al., 2014). Further, these studies found that mechanisms that depotentiate the synapse can inhibit reinstatement to cocaine seeking (Gipson et al., 2013; Shen et al., 2014). We hypothesized that D-serine augmentation activates hypofunctional NMDA receptors and depotentiates the synapse, and ultimately elicits a reduction in cocaine seeking behavior. Interestingly, D-serine augmentation increased evoked NMDA mediated receptor currents in both cocaine and saline self-administering animals. There were no changes in NMDA receptor composition or function following D-serine augmentation, as animals treated with D-serine augmentation had no difference in total protein or GluN1, GluN2A or GluN2B subunit surface expression at either basal levels or following a 1 hr reinstatement test.

I found a lack of trafficking of AMPA and NMDA receptors, revealing that during reinstatement test there is not an increase AMPA receptors at the surface causing the transient potentiation shown previously (Gipson et al., 2013; Shen et al., 2014). Further, these results indicate that the increase in evoked NMDA receptor current in D-serine augmented animals was not a result of increased NMDA receptors at the surface. These collectively lead to a picture of unchanged NAc core AMPA and NMDA receptor protein expression, but a change in potentiation of the synapse after animals are treated with D-serine augmentation. This is interesting, because despite there being no D-serine present in the wash solution we see elevated NMDA receptor mediated currents in treated animals. This could be indicative of a treatment induced reversal of subunits not explicitly investigated, such as a decrease in the cocaine-induced elevation of GluN3A (Tang et al., 2004), a subunit whose properties are associated with a reduction of induced NMDA receptor currents (Das et al., 1998; Kehoe, Bernardinelli, & Muller, 2013; Pérez-Otaño et al., 2001; Sasaki et al., 2002; Wada, Takahashi, Lipton, & Chen, 2006).

Potential cellular mechanisms of D-serine augmentation: NMDA receptor modulation of dopamine.

NAc core dopamine projections largely come from the ventral tegmental area (VTA), the source of dopamine in the mesolimbic dopamine system. Inhibition of the VTA also attenuates cocaine-induced reinstatement (McFarland & Kalivas, 2001; Shen et al., 2014) and depotentiates NAc core MSN following an acute cocaine challenge (Shen et al., 2014). Additionally, systemic administration of dopamine antagonists attenuates cocaine-induced reinstatement and blocks the transient increase in synaptic strength (Shen et al., 2014). Moreover, direct antagonism of dopamine receptors into the NAc core attenuates cue-induced reinstatement (Saunders, Yager, & Robinson, 2013). Recently, it has been found that NMDA into the NAc dose-dependently reduces dopamine transmission in a metabotropic glutamate

receptor dependent manner (Yavas & Young, 2017) and that GluN2D containing NMDA receptors are important in this effect (X. Zhang et al., 2014). Further, serine racemase knockout mice exhibit basal increases in NAc dopamine and glutamate (Puhl et al., 2017). Collectively, these data indicate that the mechanism of D-serine augmentation could be activation of NAc NMDA receptors causing a decrease in dopamine transmission.

Alternatively, PL-NAc projections are integral for cocaine seeking, and they may operate in a VTA dopamine-dependent pathway, as shown by an elegant study where cue-induced cocaine seeking was attenuated by contralateral injection of dopaminergic D1/D2 antagonist in the PL and glutamatergic ionotropic antagonism (APV and CNQX) of the NAc core (McGlinchey et al., 2016). Thus, our systemic D-serine augmentation could be counteracting dopamine descending influence in the VTA->PL->NAc core pathway, in addition to effects in the NAc, by inhibiting VTA afferents.

Prelimbic cortical (PL) glutamatergic afferents to the NAc core are integral to cue- and cocaine-induced reinstatement, but differentially regulate potentiation of NAc core MSN synapses during cue- or cocaine-induced reinstatement. PL pharmacological inactivation attenuated both cue-induced (Gipson et al., 2013) and cocaine-induced reinstatement (McFarland & Kalivas, 2001; Shen et al., 2014), however, inactivation of the PL has divergent effects on synaptic potentiation during reinstatement using different modalities. Pharmacological inactivation of the PL blocked the transient cue-induced potentiation, but facilitated cocaine-induced transient potentiation in the NAc core (Shen et al., 2014). These findings lead us to consider the fact the PL may not be driving the effect behind our cocaine plus cue-primed reinstatement test, as cue and cocaine primes have opposing effects on NAc core MSN synaptic potentiation.

Interestingly, these are not the first results to indicate divergent roles of the PL in addiction behaviors. PL activation in humans is associated with selecting appropriate behavior responses, and thus PL activation can produce contradictory results, based whether responding

or not responding is the most adaptive behavioral response. Representative of this, inhibition of the PL promoted cocaine seeking in a model of compulsive drug use (Chen et al., 2013), where PL activation is important for devaluing cocaine in the presence of punishment, and also attenuated cocaine seeking in a model of cocaine self-administration and extinction (Gipson et al., 2013; Shen et al., 2014), where the PL codes the value of cocaine-conditioned cues.

Future Directions

The experiments presented in this dissertation support the hypothesis that NMDA receptors oppose cocaine plus cue-primed reinstatement to cocaine seeking and, further, indicate D-serine treatment during abstinence could be an effective pharmacotherapeutic tool for combating relapse in psychostimulant abuse disorders. However, these experiments also pose new questions that warrant further investigation. For instance, there still needs to be a further investigation into the locus of the systemic D-serine augmentation effect and further characterization of the molecular and functional changes following cocaine self-administration, extinction training and D-serine augmentation. These are outlined in more detail below.

Behavioral Investigations

Although we give evidence that NMDA receptor activation in the NAc core is sufficient to attenuate reinstatement to cocaine seeking, we do not directly prove that the NAc is necessary for the effect of systemic D-serine augmentation. One could investigate this with an experiment where systemic 3 day D-serine augmentation is combined with a glycine modulatory site (GMS) antagonist injected into the NAc core directly before reinstatement to cocaine seeking. If the NAc core is the main locus of the systemic effect, animals injected with the GMS inhibitor will have significantly greater reinstatement responding than D-serine augmented animals injected with vehicle. It is possible that there will be no effect when the NAc is inhibited by the GMS antagonist, and this could have two meanings. First, (i) the NAc is could not be the locus of the

mechanism of the systemic D-serine augmentation, or (ii) the NAc is one of several brain areas contributing to the D-serine augmentation effect, and when the NAc is blocked other regions compensate. NMDA receptor antagonism has been shown to have bidirectional effects on reinstatement behavior (Backstrom & Hyytia, 2007; Famous et al., 2008) and is a potential confound of this experiment. Thus, antagonism of the GMS of NMDA receptors could potentiate cocaine seeking without affecting the mechanisms of D-serine augmentation and give a false negative result.

Investigations into neurotransmission during a cocaine plus cue-primed reinstatement test.

Also, data presented herein indicate that D-serine impairs cocaine plus cue-primed reinstatement, but does not address these modalities independently. We hypothesize that our cocaine plus cue-primed reinstatement test leads to an enhanced release of glutamate during the reinstatement test, compared to a reinstatement test of just one prime alone, and accordingly, that an effect on reinstatement might not be observed for cue-prime or cocaine-primed reinstatement alone. This increase in glutamate combined with D-serine augmentation, to alleviate deficits in volume transmission of endogenous D-serine, allows for enhanced activation of NMDA receptors. This can be investigated by using microdialysis to assess glutamate levels in the accumbens during a cocaine, cue or cocaine plus cue-primed tests of reinstatement and comparing glutamate levels among these tests. Of interest, a study looking at NAc core glutamate release following a meth plus cue-primed reinstatement gives support that this may be the case (Parsegian & See, 2014), however they never directly compared glutamate release between the reinstatement tests.

Further, microdialysis investigating the effects of D-serine augmentation on dopamine release during a cocaine plus cue-primed reinstatement event. It's possible that stimulating NMDA receptors with D-serine is inhibiting dopamine release in the PL and NAc core during the

reinstatement test and thus reducing reinstatement behavior, microdialysis experiments could investigate this hypothesis.

These results would advance knowledge on how cocaine and cue priming affects mechanisms of relapse, but also why our D-serine augmentation is effective only during a cocaine plus cue-primed reinstatement event and not either prime alone (data not shown).

Cellular Investigations

Our investigation into AMPA and NMDA receptor subunit expression was limited to cocaine seeking animals with or without D-serine augmentation. Additionally, we did not assess levels of the less common NMDA receptor subunits like GluN2C/D or GluN3A, that upon a further investigation of the current literature may be important in cocaine-induced adaptations in the mesolimbic dopamine system. Future experiments should investigate potential changes in these subunits in total protein and surface protein expression, as well as compare all glutamatergic iontropic receptor subunits and GLT-1 to cocaine naïve animals.

It was beyond the scope of this dissertation to investigate the transient synaptic potentiation seen following cue- or cocaine-prime (Gipson et al., 2013; Shen et al., 2014), and whether this LTP-like plasticity is present in a cocaine plus cue-primed test of reinstatement. Moreover, if there is a transient synaptic potentiation following a cocaine plus cue-primed reinstatement test it is likely that D-serine augmentation is also attenuating this synaptic change that is necessary for reinstatement of cocaine seeking (Shen et al., 2014).

Regardless of the direction these future studies will take, a further investigation into NMDA receptors opposition of cocaine seeking and the mechanism of D-serine augmentation attenuation of cocaine seeking is a promising avenue for the development of pharmacotherapeutics for psychostimulant use disorders.

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