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Role of Glutamate on Dopamine Neurochemistry and the Behavioral Pharmacology of Cocaine in Squirrel Monkeys

by

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Role of Glutamate on Dopamine Neurochemistry and the Behavioral Pharmacology of Cocaine in Squirrel Monkeys

By

Rayna M. Bauzo
B.S., Florida State University, 1998

Adviser: Leonard L. Howell, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of
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Abstract

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By Rayna M. Bauzo

Basal glutamate originating primarily from the cystine-glutamate transporter provides glutamatergic tone on extrasynaptic glutamate receptors such as the metabotropic glutamate 2/3 receptors (mGluR2/3). Glutamate has been shown to regulate dopamine function in the mesocorticolimbic pathway in rodents, which plays an important role in the behavioral pharmacology of psychostimulants. Hence, glutamate systems may be effective targets for cocaine pharmacotherapeutics. To investigate whether cystine-glutamate transporter or mGluR2/3 modulation would alter the neurochemical and behavioral effects of cocaine, NAC, a cystine prodrug, and LY379268, an mGluR2/3 agonist, were administered prior to cocaine during in vivo microdialysis and operant behavioral tasks. It was hypothesized that augmenting extrasynaptic glutamate release or mGluR2/3 activation would attenuate cocaine- or amphetamine-induced increases in extracellular dopamine and their corresponding behavioral-stimulant, reinforcing and reinstatement effects. NAC significantly attenuated cocaine-induced increases in dopamine but had inconsistent effects on amphetamine-induced increases in dopamine. However, NAC did not alter the behavioral effects of either cocaine or amphetamine. Similarly, LY379268 significantly attenuated cocaine-induced increases in dopamine. LY379268 also significantly attenuated cocaine-induced behavioral-stimulant effects but drug interactions on cocaine self-administration and reinstatement were less robust and inconsistent across pretreatment doses. Hence, drug interactions on neurochemistry were only partially reflected in behavioral measures, likely due to the incomplete blockade of cocaine-induced increase in extracellular dopamine by NAC and LY379268.

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Dedication

This work is dedicated to my baby, Ollon Antonio Bauzo Whitfield.

Before I was a mom, I had no idea of my own capabilities. When you came, I wanted to be a better person for you. I strove to be the best I could be, so that I would set a good example for you. I took a huge leap of faith to come to Emory and Atlanta, just the two of us. Tony, I would not have gotten this far without your help. I was blessed with a well-behaved and understanding boy. I am fully aware of how truly blessed I was to have you, the youngest student in my Neuroscience classes. Thank you for always reminding me that there was life outside of science and having patience with me when life had to be all about science. It wasn't an easy road, but I'm glad I took this journey with you by my side. Being your mom taught me that through perseverance I could do anything. I hope that one day I can pass this valuable lesson on to you. Always remember that you should never have a wishbone where your backbone should be.

Table of Contents

Table of Figures

Table of Tables

I. INTRODUCTION

One of the most difficult aspects of treating cocaine addiction is the high probability of relapse even after extended periods of abstinence. Cocaine binds to the dopamine transporter blocking the reuptake of dopamine which leads to enhanced dopamine receptor activation (Ritz et al., 1987). The role of dopamine in the reinforcing effects of psychostimulants is well-established, and potentiation of dopamine in the mesocorticolimbic pathway can enhance reinforcement (Di Chiara and Imperato, 1988). Because of dopamine's role in the reinforcing effects of psychostimulants, dopamine receptors and dopamine transporters have been targeted for pharmacotherapeutics of cocaine addiction.

Extrasynaptic glutamate regulates dopamine function in the mesocorticolimbic pathway (Cartmell and Schoepp, 2000). Extrasynaptic glutamate is primarily regulated by the cystine-glutamate transporter (Baker et al., 2002b). These transporters, preferentially located on glial cells in the central nervous system, exchange an extracellular cystine for an intracellular glutamate (Pow, 2001). This extrasynaptic glutamate maintains a homeostatic basal activation or tone on group II metabotropic glutamate receptors (mGluR2/3) to modulate glutamate and dopamine neurotransmission (Baker et al., 2003a; Baker et al., 2002b). Repeated cocaine administration decreases basal glutamate in rodents and down regulates cystine/glutamate transporter expression (McFarland et al., 2003). The down regulation of cystine/glutamate transporter expression may contribute further to the decrease in basal glutamate. This diminished basal extrasynaptic glutamate results in decreased activation of mGluR2/3 receptors, which in turn facilitates cocaine-induced synaptic release of glutamate and dopamine (Baker et al., 2003a; McFarland et al., 2003). Understanding how glutamate modulates dopamine via mGluR2/3 receptors may lead to

a better understanding of the mechanisms of action of cocaine and may lead to the development of better pharmacotherapies for cocaine addiction.

A. Specific aims

1. To determine the role of modulating cystine-glutamate transporters on the neurochemical and behavioral pharmacology of cocaine in squirrel monkeys. Hypothesis: Augmenting cystine-glutamate transporter activity will increase glutamatergic tone on extrasynaptic glutamate receptors and will attenuate cocaine-induced increases in dopamine and its metabolites. These neurochemical changes will be reflected by an attenuation of the behavioral-stimulant and reinforcing effects of cocaine. The cystine-glutamate transporter will be pharmacologically manipulated with a cystine prodrug, N-acetyl-L-cysteine (NAC) in squirrel monkeys. In vivo microdialysis will quantify levels of extrasynaptic glutamate and extracellular dopamine and its metabolites. Neurochemical changes will be correlated to changes in the stimulant effects of cocaine on operant behavior maintained by a fixed-interval schedule of stimulus termination. Subsequently, the reinforcing properties of cocaine will be characterized using a second-order schedule of cocaine self-administration. Lastly, self-administration behavior will be extinguished and cocaine priming injections will be used to reinstate extinguished drug self-administration behavior.

2. To determine the role of modulating mGluR2/3 on the neurochemical and behavioral pharmacology of cocaine in squirrel monkeys. In order to demonstrate that the cystine-glutamate transporter is causing its effects by altering extrasynaptic glutamate tone on mGluR2/3, these receptors will be modulated directly with a selective mGluR2/3 agonist and antagonist. Hypothesis: Augmenting mGluR2/3 signaling will diminish the neurochemical effects of cocaine, and these changes will be reflected in an attenuation of behavioral-stimulant and reinforcing effects of cocaine. The mGluR2/3 will be

pharmacologically manipulated with the mGluR2/3 agonist, LY379268, and the mGluR2/3 antagonist, LY341495. In vivo microdialysis will quantify levels of extrasynaptic glutamate and extracellular dopamine and its metabolites. Neurochemical changes will be correlated to changes in the stimulant effects of cocaine on operant behavior maintained by a fixed-interval schedule of stimulus termination. Subsequently, the reinforcing properties of cocaine will be characterized using a second-order schedule of cocaine self-administration. Lastly, self-administration behavior will be extinguished and cocaine priming injections will be used to reinstate extinguished drug self-administration behavior.

II. BACKGROUND AND SIGNIFICANCE

A. Cocaine abuse

Cocaine, a highly addictive psychostimulant, is the second most abused illicit drug in the United States, according to the Drug Enforcement Agency (DEA). Cocaine/crack use was widespread in almost all 21 areas included in the survey by the Community Epidemiology Work Group (CEWG). According to the National Survey on Drug Use and Health (NSDUH) cocaine use has remained stable since 2002, with an estimated 34.15 million Americans who tried cocaine and 2.3 million current users. The Lewin Group for the National Institute of Drug Abuse (NIDA) reported that the government spent $97.7 billion dollars on substance abuse, including treatment and prevention, crime and welfare costs, and costs related to lost wages and reduced productivity. Data from the Drug Abuse Warning Network (DAWN) showed a 33% increase in cocaine related emergency room visits between 1995 and 2002. Despite the high social, health and economic costs to the individual users as well as to society, cocaine use and abuse remains a pervasive problem.

B. Rationale for Targeting Dopamine:

Despite its serious negative impact, there are no effective treatments for cocaine addiction. Lack of effective treatments has prompted a major effort to develop pharmacological agents to treat this behavioral disorder. Understanding the mechanisms of action of cocaine is important in the development of pharmacotherapies for addiction and preventing relapse. One of the major strategies in medications development is to interfere with cocaine's actions at the dopamine transporter (DAT). Cocaine binds to monoamine transporters equipotently and blocks the reuptake of dopamine, serotonin, and norepinephrine (Ritz et al., 1990). When activated these

neurons release their respective neurotransmitter to initiate postsynaptic signaling. The monoamine transporters act as the primary inactivators of signaling by removing the neurotransmitters from the synaptic cleft. Blockade of these transporters subsequently leads to accumulation of these monoamines and potentiation of postsynaptic receptor activation. However, the behavioral-stimulant and reinforcing effects of cocaine are most attributed to the increase in dopamine following blockade of the dopamine transporter (Ritz et al., 1987).

In vivo neurochemical studies have repeatedly demonstrated a strong correlation between the behavioral effects of cocaine and an increase in extracellular dopamine, dopaminergic cell activation, or evoked dopamine in different parts of the brain including the ventral tegmental area (VTA) (Almodovar-Fabregas et al., 2002), nucleus accumbens (Di Chiara and Imperato, 1988; Pettit and Justice, 1989, 1991; Wu et al., 2001), striatum (Church et al., 1987; Garris and Wightman, 1995; Jones et al., 1996; Kimmel et al., 2005; Nicolaysen et al., 1988), prefrontal cortex (Steketee, 2003; Williams and Steketee, 2004), and amygdala (Garris and Wightman, 1995). In vivo microdialysis studies report that repeated cocaine administration induced an increase in plasma concentrations of cocaine. The increases in plasma concentrations of cocaine were consistent with increased extracellular concentrations of cocaine in the nucleus accumbens. These increases corresponded to an increase in extracellular dopamine in the nucleus accumbens in response to an acute injection of cocaine in rats (Pettit et al., 1990). Similarly in the striatum, cocaine produced a similar time course for extracellular cocaine concentration and extracellular dopamine levels, as shown in microdialysis studies. Though there was a difference in magnitude, there was a linear correlation between cocaine concentration and extracellular dopamine (Nicolaysen et al., 1988). Cocaine self-administration increased extracellular levels of dopamine above baseline levels in the nucleus accumbens of rats, and self-administration responding was titrated

to maintain stable levels of elevated dopamine (Pettit and Justice, 1989). Interestingly, response-contingent cocaine self-administration increased extracellular dopamine to a greater extent than response-independent cocaine administration (Hemby et al., 1997; Kimmel et al., 2005), implicating a role of dopamine in the underlying mechanisms of learning associated with cocaine addiction. In nonhuman primates, in vivo microdialysis also demonstrated an increase in extracellular dopamine following cocaine self-administration that is not apparent when cocaine-associated cues are presented alone (Bradberry et al., 2000). Microdialysis studies also demonstrate that after 3 days of abstinence in cocaine-treated rats, basal dopamine levels were decreased in the nucleus accumbens as compared to saline treated animals, while cocaine-induced increases in extracellular dopamine were enhanced in the nucleus accumbens (Chefer and Shippenberg, 2002). Therefore, neuroadaptive changes in dopamine signaling may be important for the long lasting propensity for relapse in cocaine abusers. Selectivity, time course of onset, and duration of action are predictive of the reinforcing properties of dopamine transporter inhibitors (Kimmel et al., 2007; Lile et al., 2003).

Real-time voltammetry studies further support the link between cocaine's effects and neurochemical effects on dopamine. In vivo voltammetry demonstrated a strong correlation between real-time increases in dopamine in the nucleus accumbens and psychomotor stimulant behavior induced by cocaine (Broderick, 1991). Consistent with microdialysis, repeated cocaine administration induced a decrease in basal dopamine in the nucleus accumbens, as well as an enhanced cocaine-stimulated increase in dopamine release and uptake (Ng et al., 1991). Interestingly, real-time fast-scan cyclic voltammetry demonstrated that both noncontingent cocaine and self-administered cocaine increased dopamine release transients at a timescale similar to cocaine uptake into the brain. However, the increases in dopamine during cocaine self-administration were evident slightly before transport of cocaine to the brain but coinciding with lever press behavior

(Stuber et al., 2005). This suggests that dopamine plays an important role in the learned association of cocaine to operant behavior and contextual cues that predict cocaine availability.

In addition to neurochemical evidence for the involvement of dopamine in the mechanism of action of cocaine addiction, there are numerous behavioral studies that support the role of dopamine in the mechanism of action of cocaine. One of the well established behavioral effects of cocaine is increased locomotor behavior and stereotypic behaviors manifested in humans by hyperactivity and repetitive motions. In rodent models, selective dopamine transporter inhibitors and dopamine agonists significantly increased locomotor activity (Heikkila and Manzino, 1984; Schindler and Carmona, 2002), whereas selective lesioning of the mesolimbic dopamine neurons blocked the ability of cocaine to induce locomotor activity (Kelly and Iversen, 1976). Selective dopamine transporter inhibitors that have a rapid rate of onset and are short acting also induce behavioral-stimulant effects in nonhuman primates (Ginsburg et al., 2005; Kimmel et al., 2007). Moreover, the selectivity and pharmacokinetic properties of dopamine transporter inhibitors at the dopamine transporters are predictive of the behavioral-stimulant properties of the inhibitors. Thus, dopamine plays a major role in the behavioral-stimulant effects of cocaine.

The drug self-administration paradigm has been established as a tool to measure abuse liability of drugs. Cocaine maintains self-administration in all animal models including simple C. elegans, rodents, nonhuman primates, and humans. The increase in extracellular dopamine has been implicated in the reinforcing properties of cocaine. Nonhuman primates readily self-administer dopamine D2 receptor agonists without the need for prior cocaine self-administration training to acquire dopamine agonist self-administration (Weed and Woolverton, 1995). The role of dopamine D1 receptors is unclear in the effects of cocaine self-administration (Woolverton, 1986). Dopamine D1

receptor agonists are self-administered, but this is dependent on the schedule of reinforcement and the selectivity of the agonist (Grech et al., 1996). Both selective dopamine D1 agonists and antagonists can attenuate cocaine self-administration and cocaine-induced reinstatement in rodents (Caine et al., 1995; Milivojevic et al., 2004; Ranaldi and Wise, 2001; Self et al., 2000; Weber et al., 2001; Weed et al., 1998). However, dopamine D1 receptor knock-out mice do not self-administer cocaine (Caine et al., 2007). Hence, both the dopamine D1 and D2 receptors play important roles in cocaine-maintained self-administration. Rodents and nonhuman primates readily self-administer cocaine analogs with similar selectivity, pharmacokinetic and pharmacodynamic profiles to cocaine (Kimmel et al., 2007; Roberts et al., 1999; Wilcox et al., 2002).

Given that dopamine signaling plays an important role in the behavioral effects of cocaine, a number of studies have focused on the dopamine transporter and dopamine receptors as pharmacological targets for cocaine addiction. Researchers have attempted to design an agonist therapy, much like methadone for heroin addicts, with little success. Current research suggests that the neuroadaptive changes in the brain due to chronic cocaine administration are complex and that research should expand beyond dopamine. Though cocaine-induced neurochemical and behavioral effects are most attributed to dopamine, there is growing evidence for the involvement of multiple neurotransmitter systems in the underlying mechanisms of cocaine-induced neuroplastic changes, as well as modulatory signaling on dopamine, including glutamate.

C. Rationale for targeting Glutamate:

The glutamate system has been linked to locomotor sensitization, conditioned place preference, and self-administration of drugs typically abused by humans such as cocaine and morphine (Carlezon and Nestler, 2002; Cornish and Kalivas, 2000, 2001;

Pierce et al., 1996), all of which are procedures often used to model drug abuse in rodents. Glutamate is the major excitatory neurotransmitter in the central nervous system and numerous studies demonstrate direct interactions between glutamate and dopamine. Glutamate has been demonstrated to provide a tonic excitatory effect on dopamine release. When the glutamatergic tone is blocked, the ability of dopamine transporter blockers to enhance extracellular dopamine is attenuated (Moghaddam and Bolinao, 1994). Exogenous application of glutamate into the striatum has led to inconsistent effects on dopamine release, however glutamate antagonists attenuate basal dopamine further supporting an excitatory tonic glutamate tone on dopamine neurons (Kulagina et al., 2001). Moreover, pharmacological stimulation of glutamatergic afferents from the hippocampus and subthalamic nuclei on dopamine neurons resulted in an increase in extracellular dopamine (Ampe et al., 2007; Peleg-Raibstein et al., 2005). Thus, the tonic excitatory effect of glutamatergic input on dopamine neurons has been well established. In contrast, electrophysiological and microdialysis studies demonstrated a tonic inhibitory influence of dopamine neurons on glutamate activation (Ampe et al., 2007; Mulder et al., 1996; Porras and Mora, 1995; Yamamoto and Davy, 1992). In vivo electrophysiological studies reported that selective dopamine depletion led to an enhanced firing rate of glutamatergic neurons (Mulder et al., 1996). Interestingly, studies have shown that dopamine agonists induce a significant increase in extracellular glutamate (Porras and Mora, 1995). Thus, glutamate and dopamine have complex reciprocal signaling that may be important for the underlying mechanisms of neurological disorders.

Though cocaine exerts pharmacological effects on monoamine transporters, many studies have demonstrated that cocaine also has pharmacological effects on extracellular glutamate. Both contingent and noncontingent cocaine administration increased extracellular glutamate in a number of glutamate rich areas of the brain

including the ventral pallidum (Sizemore et al., 2000), nucleus accumbens (McFarland et al., 2003; Pierce et al., 1996; Smith et al., 1995), and medial prefrontal cortex (Reid et al., 1997; Williams and Steketee, 2004). Studies in rodents reported that after chronic cocaine administration and withdrawal, a subsequent cocaine injection induced a sensitized glutamatergic response (Pierce et al., 1996; Reid and Berger, 1996). However, Pierce et al. (Pierce et al., 1996), reported that the enhancement of cocaine-induced increases in extracellular glutamate only occurred in subjects that also demonstrated behavioral sensitization. In contrast to these reports, (McKee and Meshul, 2005) reported that a single injection of cocaine did not have an immediate effect on extracellular glutamate, but instead increases in glutamate were only visible 1 day post acute injection. Interestingly, this same report demonstrated a long term attenuation of extracellular glutamate after an acute injection of cocaine that was seen 3 days to at least 2 weeks after the injection. This is similar to reports demonstrating a decrease in basal glutamate after chronic administration of cocaine (Baker et al., 2002a; Miguens et al., 2008a; Miguens et al., 2008b). Interestingly, both dopamine depletion and dopamine antagonists (D1/D2) blocked the ability of cocaine to increase extracellular glutamate (Reid et al., 1997), suggesting that the effect of cocaine on glutamate is mediated by the dopaminergic effects of cocaine. Accordingly, cocaine-induced changes in extracellular glutamate have been implicated in the underlying mechanisms of the behavioral effects of cocaine.

The long term behavioral effects of cocaine such as sensitization, reinstatement and conditioned place preference may be mediated through glutamatergic signaling. In rodents, sensitization has been used to show the neuroplastic adaptations caused by chronic cocaine administration. Chronic cocaine administration leads to a long-lasting enhanced cocaine-induced locomotor activation after a period of withdrawal. Removing the glutamatergic signal via prefrontal cortex lesions or glutamate antagonists blocks the

induction of cocaine-induced behavioral-stimulant effects (Li et al., 2000; Witkin, 1993). Thus, glutamate activation is essential for the expression of behavioral sensitization. Moreover, glutamatergic inputs to the VTA are also necessary for the reinforcing effects of cocaine. Blockade of VTA glutamate receptors prevents cocaine-induced place preference (Harris and Aston-Jones, 2003), which is a model used to measure the rewarding properties of cocaine in rodents. Glutamate has also been shown to play an integral role in reinstatement of extinguished self-administration in rodents. Cocaine-induced reinstatement of extinguished self-administration is dependent on increased glutamatergic transmission from the prefrontal cortex. Extracellular glutamate is enhanced during cocaine primed reinstatement. Both the rise in extracellular glutamate and reinstatement were blocked by selectively inactivating the dorsal prefrontal cortex (McFarland et al., 2003). Also, cocaine administered directly into the prefrontal cortex induced reinstatement via increased glutamatergic signaling on the nucleus accumbens (Park et al., 2002). Reinstatement of extinguished self-administration by stimulation of hippocampal glutamatergic neurons was dependent on glutamate into the VTA (Vorel et al., 2001). Together, these data suggest that glutamate is an essential component of the long-lasting behavioral effects of cocaine.

D. Cystine-glutamate transporter

The cystine-glutamate transporter is a membrane bound transporter that is part of the di-sulfide-linked heteromeric amino acid transporter family designated system X_c^- (Sato et al., 1999). The transporter is made up of a novel protein, xCT, and a heavy chain, 4F2hc, common to other transporters of this family (Sato et al., 1999). Unlike the X_{AG-} superfamily, which is Na^+-dependent and functions to remove glutamate from the extracellular space, the cystine-glutamate transporter is a Na^+-independent, anionic amino acid transporter that releases glutamate into the extracellular space. This

transport system is established as an obligatory exchange with a molar ratio of 1:1 and exchanges one extracellular cystine for one intracellular glutamate (Patel et al., 2004). It is important for maintaining glutathione levels, as cysteine is the rate-limiting substrate for making glutathione. Glutathione is the major cellular antioxidant and its depletion can leave neurons susceptible to oxidative stress and neuronal death. This tripeptide is made up of glutamate, glycine and cysteine. Both glutamate and glycine are ubiquitous in cells, however cysteine is not. Thus, the role of the cystine-glutamate transporter in preventing oxidative stress has been well established.

Extracellularly, both cysteine and glutamate are excitotoxic and extracellular concentrations are regulated. L-cysteine is unstable as a free amino acid and is readily broken down in the blood and potentiates glutamate toxicity. Therefore, it is readily oxidized to cystine (Puka-Sundvall et al., 1995). However, once inside the reductive environment of the cell, cystine is reduced to cysteine. Thus the extracellular concentration of cystine is approximately 100-fold greater than the intracellular concentration (Murphy et al., 1989). Extracellular glutamate is tightly maintained and rapidly taken up by the X_{AG-} superfamily of Na^+-dependent glutamate transporters. The glutamate intracellular concentration is 10,000-fold greater than the extracellular compartment. Therefore, the transporter exchanges glutamate and cystine passively down the concentration gradients. The cystine prodrug, N-acetyl-L-cysteine (NAC), enhances extracellular concentrations of cystine and therefore enhances the uptake of cystine and the release of glutamate. In the extracellular compartment, NAC is deacylated to the amino acid L-cysteine, which is readily oxidized to L-cystine. NAC is excitotoxic in cultured cells, but in vivo excitotoxicity has yet to be demonstrated. Thus, NAC is a suitable pharmacological tool for the manipulation of the cystine-glutamate transporter.

The cystine-glutamate transporter is ubiquitously expressed throughout the brain. Though the transporters are primarily localized on glial cells, particularly in astrocytes, these transporters are also localized on neuronal cells (Burdo et al., 2006; Pow, 2001; Sato et al., 2002). Further supporting the role of the transporter in preventing oxidative stress, these transporters are highly expressed on mitochondrial complexes (Sato et al., 2002). These transporters are distributed throughout the brain, however they are densely expressed in the circumventricular organs (Burdo et al., 2006). This is most likely to prevent oxidative stress from agents crossing the blood-brain barrier. Cocaine has been reported to induce oxidative stress (Dietrich et al., 2005; Pacifici et al., 2003). Thus, manipulating the transporter would be an appropriate target to investigate the effects of cocaine. However the role of the cystine-glutamate transporter in maintaining extracellular glutamate became the primary motivation in investigating the role of the transporter in the behavioral effects of cocaine.

Extracellular glutamate plays a major role in the neuroplastic changes underlying cocaine addiction. Dysregulation of basal glutamate has also been implicated as an important component of cocaine-induced reinstatement. An acute cocaine injection can increase both dopamine and glutamate synaptic release in the nucleus accumbens (McFarland et al., 2003). In contrast, repeated cocaine administration decreases basal extrasynaptic glutamate (Baker et al., 2002a). Extracellular glutamate can be regulated by vesicular release of glutamate (Baker et al., 2002b). However, basal extracellular glutamate seems to primarily originate from cystine/glutamate exchange via transporters located primarily on astrocytes (Baker et al., 2002b; Pow, 2001). This extrasynaptic glutamate provides tonic regulation on extrasynaptic glutamate receptors, including the metabotropic glutamate receptors. Dopamine function in the mesocorticolimbic pathway is regulated via tonic regulation of group II metabotropic receptors (mGluR2/3) (Baker et al., 2003a; Baker et al., 2002b). These receptors act as

autoreceptors to regulate pre-synaptic neurotransmitter release. Repeated cocaine administration decreases basal glutamate in rodents (McFarland et al., 2003), which results in decreased activation of mGlu2/3 receptors, which in turn facilitates synaptic release of glutamate and dopamine. Understanding the mechanisms by which glutamate modulates dopamine in the context of cocaine neuropharmacology may uncover new targets in developing pharmacotherapeutic medications for cocaine addiction.

E. Metabotropic glutamate receptors

In addition to dopamine receptors, populations of glutamate receptors are located in the nucleus accumbens and other limbic areas important for psychiatric disorders and drug addiction. Glutamate receptors can be divided into two families, ionotropic and metabotropic receptors (Conn and Pin, 1997; Nakanishi, 1994a, b, 1992; Nakanishi and Masu, 1994; Nakanishi et al., 1994; Nakanishi et al., 1998; Nakanishi et al., 1996). Ionotropic receptors include NMDA receptors and the non-NMDA receptors (AMPA/Kainate) and utilize ion channels to mediate fast synaptic transmission throughout the central nervous system. Because of the ubiquitous involvement of these receptors in excitatory processes in the central nervous system, targeting these receptors leads to side effects that can be detrimental. Thus these receptors are not considered ideal targets for therapeutics. On the other hand, metabotropic glutamate receptors have the capacity to modulate the actions of the ionotropic glutamate receptors as well as other neurotransmitter receptors making them ideal candidates for pharmacotherapeutic targets (Pin and Bockaert, 1995; Pin and Duvoisin, 1995).

The metabotropic glutamate receptors share very little sequence homology with the G-protein coupled receptor (GPCR) superfamily and thus along with a Ca^{2+} sensing receptor and the $GABA_B$ receptor make up a new family of GPCR, the GPCR family 3. These receptors are characterized by an extracellular N-terminal domain, the typical

GPCR seven transmembrane domains, and an intracellular C-terminal tail of variable length (Bockaert and Pin, 1999; Conn and Pin, 1997). Within this receptor family, 19 cysteine residues in the extracellular domain and extracellular loops are conserved and may play a role in either the three dimensional structure of the molecules or the intramolecular transduction mechanisms (Conn and Pin, 1997). Unlike other GPCRs, the binding site for the mGluRs is not located in the pocket formed by the seven transmembrane domains, instead it is found on the extracellular domain made up of two globular domains with a hinge region. This binding domain structure for agonist recognition is also found on AMPA receptors and may be common for all glutamate receptors. Further distinguishing the mGluRs from other GPCR families is the localization of the G-protein coupling domain. In other families, the coupling domain is on the third intracellular domain, while in the mGluRs, the second intracellular domain plays a role in the interaction between the receptors and their second messenger molecules. Thus, the mGluRs form a distinct family of GPCRs.

Eight metabotropic glutamate receptors have been cloned to date and are divided into three groups, based on sequence homology, localization, and pharmacology (Conn and Pin, 1997; Kenny and Markou, 2004). Within groups, mGluRs share approximately 70% sequence homology (Conn and Pin, 1997). Group I receptors, mGluR1 and 5, are typically located postsynaptically and couple to G_q to stimulate phospholipase C. Group II receptors, mGluR2 and 3, and Group III (mGluR 4, 6, 7 and 8) stimulate G_i and inhibit adenyl cyclase activity. Group II receptors are located both presynaptically and postsynaptically and on glia, while group III receptors typically are located presynaptically. Group II and III can be pharmacologically distinguished by differing affinities to commercially available agonists and antagonists. In rats, mGluR2/3 are highly expressed in many regions of the brain including the prefrontal cortex, VTA, nucleus accumbens and striatum, areas which have been shown to play a pivotal role in

addiction (Neki et al., 1996; Ohishi et al., 1994; Ohishi et al., 1993a, b; Petralia et al., 1996; Testa et al., 1998; Xi et al., 2002a). These receptors are localized on glutamatergic neurons that project into the VTA such as the prefrontal cortex and the interpeduncular nucleus and pontine nuclei, as well as areas that project into the nucleus accumbens such as the VTA, amygdala, hippocampus and prefrontal cortex (Neki et al., 1996). Localization of these receptors makes these receptors ideal candidates for targeting pathways involved in cocaine addiction with increased specificity.

Activation of mGluR2/3 receptors results in an attenuation of extracellular glutamate, but the effects on extracellular dopamine are inconsistent. The mGluR2/3 receptors bear an extensive endogenous tone and blockade of these receptors leads to an enhancement of extracellular glutamate. However, activation of these receptors leads to an attenuation of extracellular glutamate (Xi et al., 2002a). The mGluR2/3 receptors attenuate excitatory neurotransmission by inhibiting presynaptic release and by hyperpolarizing the postsynaptic cell (Muly et al., 2007). In addition, studies have demonstrated a significant glutamatergic tone on mGluR2/3 receptors to suppress dopaminergic release in the nucleus accumbens (Hu et al., 1999). Furthermore, activation of mGluR2/3 receptors has been demonstrated to both decrease basal dopamine and the magnitude of dopamine response to systemic phencyclidine administration in the nucleus accumbens of rats (Greenslade and Mitchell, 2004). However, mGluR2/3 activation has also been demonstrated to increase basal dopamine in the nucleus accumbens, striatum and prefrontal cortex of rats (Cartmell et al., 2001, 2000b; Cartmell et al., 2000c). Thus, the mechanisms by which mGluR2/3 modulate extracellular dopamine is complex. Nevertheless, activation of mGluR2/3 has been shown to inhibit the neurochemical and behavioral effects of cocaine.

Chronic administration of cocaine decreases basal levels of extracellular glutamate in the nucleus accumbens of rodents (Baker et al., 2003a; Bell et al., 2000;

Hotsenpiller et al., 2001; Pierce et al., 1996). An attenuation of basal glutamate has been associated with an enhanced release of glutamate by an acute infusion of cocaine. The observed augmentation of glutamate release may be due to the decreased capacity of presynaptic mGluR2/3 to inhibit glutamate release. Thus, dysregulation of basal extracellular glutamate may play an important role in cocaine-elicited behaviors (Baker et al., 2003a). Furthermore, stimulation of mGluR2/3 attenuated amphetamine-induced increases in locomotor activity, while blockade of mGluR2/3 enhanced amphetamine-induced locomotor activity (O'Neill et al., 2003). Moreover, amphetamine self-administration in rats (Kim et al., 2005) and cocaine self-administration in nonhuman primates (Adewale et al., 2006) was attenuated by selective mGluR2/3 agonists. In rats, cue-induced reinstatement of heroin self-administration (Bossert et al., 2005) and cue-induced reinstatement of cocaine self-administration (Lu et al., 2007) was attenuated by systemic administration of the mGluR2/3 agonist, LY379268. Additionally, drug-primed reinstatement of cocaine self-administration in rats (Baptista et al., 2004; Peters and Kalivas, 2006) and nonhuman primates (Adewale et al., 2006) was attenuated by selective mGluR2/3 activation. Therefore, it may be therapeutically beneficial to reduce glutamatergic signaling by targeting these receptors.

F. Pathways

Anatomical substrates for the interactions between dopamine and glutamate have been established in rodents (Sesack et al., 2003). The mesocorticolimbic system has been well established to play a critical role in the reinforcing and stimulant effects of cocaine. The mesocorticolimbic system has two dopaminergic pathways: one projecting from the VTA to the nucleus accumbens (mesolimbic) which is most attributed to reinforcement and another projecting from the VTA to the prefrontal cortex (mesocortical) which is involved with cognitive functions (Di Chiara and Imperato, 1988;

Hyman, 1996). Though dopamine signaling has functions independent of glutamate, glutamate often regulates critical modulatory functions of dopamine. Both the VTA and nucleus accumbens contain glutamate receptors and receive input from glutamatergic afferents (Tzschentke and Schmidt, 2003). Glutamatergic afferents from the prefrontal cortex and the laterodorsal/pedunculopontine tegmentum innervate dopamine neurons (Charara et al., 1996; Sesack et al., 2003). The regulation by these neurons is critical for dopamine's phasic firing to signal behavioral events (Sesack et al., 2003). The nucleus accumbens receives glutamatergic input from forebrain areas such as the prefrontal cortex, hippocampus and amygdala (Groenewegen et al., 1999; O'Donnell and Grace, 1995; Pennartz et al., 1994). Activation of dopamine cell bodies in the VTA result in a decrease in the accumbal response to these excitatory inputs. This suggests that the role of dopamine is to modulate the effects of excitatory afferent inputs (Brady and O'Donnell, 2004). Glutamate release in the nucleus accumbens may be modulated by presynaptic dopamine receptors (Kalivas and Duffy, 1997). Interestingly, stimulation of the hippocampal projection to the nucleus accumbens can increase VTA firing rates (Floresco et al., 2001). Moreover, stimulation of the basal lateral amygdala enhances glutamate-dependent dopamine release in nucleus accumbens (Floresco et al., 1998; Howland et al., 2002). Thus, the interactions between glutamate and dopamine are reciprocal. Glutamatergic inputs from the prefrontal cortex to the VTA synapse specifically on dopaminergic cells projecting from the VTA to the prefrontal cortex and GABAergic cells projecting from the VTA to the nucleus accumbens. This specificity of innervation may be indicative of potential mechanisms for the prefrontal cortex to modulate mesocortical and mesolimbic pathways independently by targeting dopamine or GABA receptors (Carr and Sesack, 2000). Midbrain dopamine neurons also project to the cerebral cortex and synapse on both glutamatergic pyramidal cells and GABAergic medium spiny neurons and interneurons in both rodents and nonhuman primates.

Understanding this reciprocal signaling may be important for developing pharmacotherapies of cocaine addiction.

III. MATERIALS AND METHODS

A. GENERAL METHODS

1. Subjects.

a. Nonhuman Primates

Thirty-two male adult squirrel monkeys (Saimiri sciureus) (5-15 years) weighing between 800-1200g served as subjects. Between experimental sessions, subjects were individually housed and allowed access to food twice daily (Harlan Teklad monkey chow; Harlan Teklad, Madison, WI; fresh fruit and vegetables) and had access to water ad libitum. All subjects had served in previous experiments involving acute administration of drugs. These studies were conducted in strict accordance with the NIH "Guidelines for the Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee of Emory University.

b. Rodents

Sixteen male adult Sprague-Dawley rats (8-10 weeks) weighing between 300-350g served as subjects. Between experimental sessions, subjects were individually housed in ventilated rat cages and maintained on a 12-hr light dark cycle (lights on from 7 a.m. to 7 p.m.). Subjects were allowed access to standard laboratory chow and water ad libitum. All subjects were previously drug naïve. These studies were conducted in strict accordance with the NIH "Guidelines for the Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee of Emory University.

2. Drugs.

Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD), amphetamine H_2SO_4 and N-acetyl-L-cysteine (NAC, Sigma Aldrich Corporation, St. Louis, MO) were dissolved in 0.9% saline. For reverse dialysis experiments, D,L-threo-β-hydroxyaspartic acid (THA, Sigma Aldrich Corporation, St. Louis, MO) and NAC were dissolved in aCSF. LY341495 (Tocris, Ellisville, MO) was dissolved in 0.1 N NaOH and sterile water. LY379268 (generously supplied by Dr. Jeffrey Witkin, Dept. of Psychiatry, Eli Lilly, Indianapolis, Indiana) was dissolved in sterile water. Drug doses were determined as salts. All i.m. drug injections were administered into the thigh muscle at a volume of 0.4-0.8 ml.

Table 1 – Drugs and Targets

Drug	Chemical Name	Pharmacological Target	Source
Cocaine	methyl (1R,2R,3-s,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate	Non-selective monoamine transport inhibitor	NIDA
Amphetamine	1-phenylpropan-2-amine	Non-selective monoamine transport releaser	Sigma-Aldrich
NAC	N-acetyl-L-cysteine	Augments cystine/glutamate transporter exchange	Sigma-Aldrich
THA	D,L-threo-β-hydroxyaspartic acid	Non-selective glutamate reuptake transporter inhibitor	Sigma-Aldrich
LY379268	(-)-2-oxa-4-aminobicyclo[3.1.0] hexane-4,6-dicarboxylate	mgluR2/3 agonist	Lilly
LY341495	(2-s)-2-amino-2-[1S,2-s-2-carboxycyclopronan-1-yl]-3-[xanth-9-yl]propanoic acid	mGluR2/3 antagonist	Tocris

3. Statistical analyses

The time-course data from the neurochemical studies and the stimulus termination studies and the overall rate data for the self-administration and reinstatement studies were each analyzed using repeated-measures ANOVAs. When a significant main effect was observed, Tukey's post hoc multiple comparisons tests were used to determine statistical significance, defined at the 95% level of confidence ($p < 0.05$). A one-way ANOVA was used to determine the statistical significance when comparing the glutamate responses following direct infusions of the cystine prodrug, NAC and the glutamate reuptake inhibitor, THA. The data for the direct administration of NAC in the nucleus accumbens did not meet the assumption of normality for a one-way ANOVA and therefore was analyzed using repeated measures ANOVA on ranks (chi squared test). A one-way ANOVA was also used to determine the statistical significance when comparing the peak dopamine responses following an LY379268 pretreatment. In the neurochemical data, the peak effects were determined by an obvious maximal effect or the first data point of a plateau. For the LY379268 experiments, the overall rate data for the self-administration studies were analyzed using a Mann-Whitney nonparametric test because these data did not meet the assumptions of equal variance and normality for an ANOVA.

B. METHODS USED IN MICRODIALYSIS STUDIES – NONHUMAN PRIMATE

1. Apparatus

Experimental sessions were conducted weekly in a ventilated, sound-attenuated chamber in which each monkey was seated comfortably in a commercially available primate chair (Modular Primate Chair, Med Associates Inc, St. Albans, VT). The chair restricted the movement of the animal and facilitated connection between the implanted

microdialysis probes and the microinfusion pump located outside the chamber. Typically, in vivo microdialysis sessions were limited to 5-hr and were conducted no more frequently than once per week in individual animals.

2. Guide cannula implantation

Subjects were implanted with bilateral guide cannulae (CMA/11, CMA/Microdialysis, North Chelmsford, MA) using sterotaxic procedures to target the caudate nuclei utilizing sterile surgical procedures (Czoty et al., 2000). Coordinates for the stereotaxic procedures were obtained from a standard squirrel monkey atlas (Gergen et al., 1962) (from the earbar: A/P±15.0, L/M±3.0). The cannulae were implanted flush to the skull. The subjects were initially sedated using a cocktail of ketamine, atropine, and telazol to prepare the animal for surgery and to position the subject's head in the stereotaxic frame. Anesthesia was maintained with isoflurane (1-2%) during the surgery. A drill was used to make a small burr hole directly above the caudate nucleus, and the guide cannulae were inserted to the appropriate depth. Six nylon screws (2.4 mm, Plastics One, Inc., Roanoke, VA) were implanted surrounding the guide cannulae and the preparation was secured in place with dental cement (Plastics One, Inc., Roanoke, VA). Subsequently, the skin around the implant site was sutured closed. After the surgery, a stainless steel stylet was placed in each guide cannulae to protect the site when not in use. Subjects were allowed two to three weeks for recovery before the experiments were initiated. If there was not a dopamine response to high-potassium aCSF in consecutive experimental sessions, the site was deemed nonviable and the subject was excluded from the analysis.

3. Verification of guide cannula placement

At the end of the microdialysis experiments, one subject was euthanized for verification of guide cannula placement by histology as previously described (Galvan et

al., 2003). The subject was deeply anesthetized with an overdose of pentobarbital (100 mg/kg, i.v.) and perfused transcardially with Ringer's solution, followed by 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). The brain was removed from the skull and cut into 10-mm thick blocks in the frontal plane that were further sectioned into 60-mm slices with a freezing microtome and collected in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Sections of the caudate area that contained microdialysis probes tracts were stained with cresyl violet. Based on the histology, the coordinates were deemed to be accurate for the proper placement of the cannula. In all other subjects, accurate placement of cannula was confirmed by the neurochemical profile of dopamine.

4. Experimental procedures

Microdialysis experiments were conducted in a ventilated, sound-attenuated chamber in which each monkey was seated comfortably in a commercially available primate chair (Modular Primate Chair, Med Associates Inc, St. Albans, VT) (Czoty et al., 2002; Czoty et al., 2000). The chair limited the movement of the animal and facilitated connection between the probe and perfusion pump located outside the chamber. Teflon tubing passed through the ceiling of the chamber to connect the probe implanted in the monkey to the perfusion pump. In order to prevent the subject from disturbing the probe or tubing, a Lexan plate was positioned perpendicularly to the medial plane of the subject's body just above the shoulders. Prior to surgery, the animal was acclimated to the chair and Lexan plate over several months. Commercially available probes (CMA/11) with a shaft length of 14 mm and active dialysis membrane measuring 4 x 0.24 mm were inserted into the guide cannulae. A Harvard PicoPlus microinfusion pump continuously flushed artificial cerebrospinal fluid (1.0 mM Na_2HPO_4, 150 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.15 mM ascorbic acid) through the probes via FEP Teflon

tubing to the probe at a flow rate of 2.0 μL/min for the duration of the experiment. Following a 60-min equilibration, four consecutive 10-min samples were collected for determination of baseline dopamine concentration. Twelve consecutive 10-min samples were collected after administering saline, cocaine, or amphetamine drug challenges. For 3-hr pretreatments, the drug was administered in the homecage 3-hr prior to the collection of baseline samples. For 30-min pretreatments, the drug was administered after the collection of the four baseline samples and three 10-min samples were collected following the pretreatment. Subsequently, saline or cocaine was administered. Samples were collected every 10-min in microcentrifuge tubes and immediately refrigerated.

Small-bore, high-performance liquid chromatography (HPLC) and electrochemical detection quantified levels of dopamine according to previously established protocols (Czoty et al., 2002; Kimmel et al., 2005). The HPLC system consisted of a small-bore (3.2 mm i.d. x 150 mm, 3μm) column (ESA, Inc., Chelmsford, MA) with a commercially available mobile phase (ESA, Inc.) delivered by an ESA 582 solvent delivery pump at a flow rate of 0.6 ml/min. After loading onto the refrigerated sample tray, samples (20 μl) were automatically mixed with 3 μl of ascorbate oxidase, and 5 μl of the mixture was injected into the HPLC system by an ESA model 542 autosampler. Samples were analyzed within 12 h of collection, remaining either in a refrigerator or in the refrigerated autosampler tray during this time. Electrochemical analyses were performed using an ESA dual-channel analytical cell (model 5040) and guard cell (model 5020, potential=350 mV) and an ESA Coulochem II detector. The potential of channel 1 was set to -150 mV for oxidation, while the potential of channel 2 was set to 275 mV for reduction. A full range of dopamine standards (0.5-25 nM) was analyzed before and after each set of samples to evaluate possible degradation of dopamine. Levels of dopamine below 0.5 nM were considered below the limit of detection. A desktop computer collected data and chromatograms were generated by

EZChrom Elite software (version 3.1, Scientific Software, Pleasanton, CA). The chromatograms were analyzed using the EZChrome software, comparing the experimental samples with the standards. The neurochemical effects of the drugs were compared with the neurochemical effects of saline and cocaine. Basal levels of dopamine were between 3 and 5 nM, unadjusted for probe recovery, as reported in earlier studies.

Extracellular glutamate was quantified by the Yerkes National Primate Center Biomarker Core utilizing chemically enhanced HPLC with tandem mass spectrometry (LC/MS/MS). The dansyl chloride derivatization of glutamate enhances the sensitivity of quantifying molecules using electrospray ionization mass spectrometry with the advantage of unambiguous identification of the detected analyte. Samples were first thawed just before the experiment. In a 13 X 100 test tube, 10 µL of each sample was added to the derivatization cocktail [10 µL HPLC grade water, 20 µL 0.1M $NaHCO_3$, and 5 µL internal standards solution (50 µM d6-GABA (Sigma Aldrich Corporation, St. Louis, MO) and 50 µM 5C13-Glutamate (Cambridge Isotopes)]. A 20µL dansyl chloride solution (20mg/mL in acetonitrile) was added to each test tube and samples were then corked and vortexed. The samples were then reacted at 80°C for 30 min, and then dried under nitrogen at 37°C for 30 min. The dried samples were resolubilized in 50 µL 50:50 water:acetonitrile solution. Samples were transferred to a 384 well plate to process in the mass spectrometer.

HPLC analysis was performed utilizing a Surveyor MS Pump Plus (Thermo Fisher Scientific), Micro AS autosampler (Thermo Fisher Scientific) (Timperio et al., 2007). The stationary phase of the HPLC consisted of a Discovery BioWide Pore C_{18} 15 cm X 1 mm, 5 µm packed column. The mobile phase consisted of distilled water (DD) formic acid 0.1% (v/v) (mobile phase A) and acetonitrile formic acid 0.1% (mobile phase B). The elution rate was 75% A and 25% B with a linear gradient 75% A and 50% B, with a flow rate of 75 µL/min for 7.5 min. The ESI-MS experiments (positive ion mode) were

performed utilizing a LTQ Orbitrap (Thermo Fisher Scientific). There were 4 product ion scan events of $[M+H]^+$=386.2 (labeled glutamate), 381.2 (sample glutamate), (Gabard and Mascher), 343.2 (labeled GABA) and 337.1 (sample GABA).

C. METHODS USED IN MICRODIALYSIS STUDIES – RODENTS

1. Apparatus

Experimental sessions were conducted over the course of one week in rat microisolator cages provided with microdialysis equipment (Instech Laboratories, Inc.). Microdialysis probes were connected to a Harvard PicoPlus microinfusion pump through PE-20 tubing and an ultra-low torque dual channel liquid swivel (Model 375/D/22QM, Instech Laboratories, Inc., Plymouth Meeting, PA) to allow freedom of movement. Typically, the experimental sessions were approximately 7 hr in duration and were conducted only once per subject.

2. Guide cannula implantation

Subjects were implanted with unilateral guide cannulae (CMA/12, CMA/Microdialysis, North Chelmsford, MA) using sterotaxic procedures to target the striatum utilizing aseptic techniques. Rats were anesthetized under isoflurane (1-2%) and depth of anesthesia was determined by toe pinch. Anesthesia was maintained on isoflurane (1-2%) throughout the procedure. Coordinates for the stereotaxic procedures were obtained from a standard rat atlas. Stereotaxic coordinates for the cannulae placements were +0.5 mm anterior and +3.4 mm mediolateral to bregma and -4.0 mm ventral from the surface of the skull. A small incision was made in the skin overlying the skull and Bregma was exposed for subsequent determination of the cannulation sites. Five holes were drilled in the skull (1 for the unilateral cannula and 4 for anchor screws). The cannula was lowered to the DV coordinates and jeweler's screws were anchored to

the skull and the preparation was secured with dental acrylic. Stylets were inserted once the dental acrylic hardened and left in the cannula until the microdialysis experiment. Banamine (2.5 mg/kg, s.c.) was administered immediately following surgery. Subjects were allowed a week for recovery before initiating the microdialysis experiments.

3. Verification of guide cannula placement

Following testing, all subjects were euthanized with CO2 from a compressed gas source in a clear chamber. Brains were removed, snap frozen and stored at -80˚C until further processing. Brains were sectioned at 20 μm and stained with cresyl violet for cannulae placement verification using the atlas of Paxinos and Watson (1986).

4. Experimental procedures

Microdialysis experiments were conducted in a rat microisolator cage provided with microdialysis equipment (Instech Laboratories, Inc.). Commercially available probes (CMA/12) with a shaft length of 14 mm and active dialysis membrane measuring 4 x 0.5 mm were inserted into the guide cannulae 24-hr prior to the start of the experiment. The morning of the experiment, microdialysis probes were connected to a Harvard PicoPlus microinfusion pump through PE-20 tubing and an ultra-low torque dual channel liquid swivel (Model 375/D/22QM, Instech Laboratories, Inc., Plymouth Meeting, PA) to allow freedom of movement. Artificial cerebrospinal fluid (147 mM NaCl, 2.2 mM $CaCl_2$ and 4 mM KCl) was continuously pumped through the probes via PE-20 tubing to the probe at a flow rate of 0.6 μL/min for the duration of the experiment. Following the start of dialysis perfusion, rats were left undisturbed for 120-min to allow for equilibration. Three consecutive 20 min samples were collected for determination of baseline glutamate concentration. Following collection of baseline samples, saline or NAC was administered. Samples were collected every 20 min for 4 hr

in microcentrifuge tubes and immediately frozen on dry ice. Samples were later analyzed via HPLC with tandem mass spectrometry as previously described.

D. METHODS USED IN BEHAVIORAL STUDIES

1. Apparatus

Experimental sessions were conducted daily in a ventilated, sound-attenuated chamber in which each monkey was seated comfortably in a commercially available primate chair (Modular Primate Chair, Med Associates Inc, St. Albans, VT). The chair restricted the movement of the animal and facilitated connection between the implanted catheter and the appropriate injection equipment located outside the chamber. The front wall of the chair facing the monkey was equipped with a response lever and red and white lights. The response lever registered responses monitored by computers and integrated circuitry interfaced with the chambers. Typically, operant behavioral sessions lasted one hour and were conducted five days a week. Drug time course determinations lasted one and a half hours.

2. Catheter implantation

Subjects (n=4 per group) involved in behavioral protocols were surgically implanted with an indwelling chronic intravenous (i.v.) polyvinyl chloride catheter (0.38 mm ID; 0.76 mm OD) to administer drugs intravenously. The procedure for the surgery was done according to a previously published protocol (Howell and Wilcox, 2001). Subjects were anesthetized with 1 mg/kg atropine, 20 mg/kg ketamine, and 2 mg/kg telazol. Anesthesia was maintained with ketamine supplements during the surgery. The catheter was implanted in either a right or left femoral or external jugular vein to the level of the right atrium using sterile surgical techniques. The catheter was routed subcutaneously to the interscapular region and exited the skin under a protective nylon-

mesh jacket. The catheter was filled with heparinized saline and sealed with a stainless-steel obturator when not in use. Subjects were allowed to recover for a week before beginning any experiments. If the catheter became occluded or damaged, the catheter was removed and another catheter was implanted in the same vein if possible, or in another vein.

3. Schedules of reinforcement

a. Fixed-interval stimulus termination

Each animal was trained on a 300-second (s) fixed-interval (FI) schedule of stimulus termination with a 3-s limited hold (LH). During the 300-s FI, the chamber was illuminated with a red light. After the interval elapsed, the animal had 3 s to respond once by pressing a response lever to extinguish the red light associated with an impending electric stimulus. When the animal pressed the lever during the 3-s LH and extinguished the red light, a white light was illuminated for 15 s followed by a 60-s time-out. In the absence of response in the 3-s LH, the animal received a brief (200 ms) 4 mA stimulus to a shaved area near the tip of the tail. For drug time-course experiments, each session consisted of 15 consecutive FI 300-s components, each followed by a 60-s timeout lasting approximately 90 min. This schedule has been used to reliably characterize the behavioral-stimulant effects of drugs (Ginsburg et al., 2005). For cumulative dosing experiments, each session consisted of 13 consecutive FI 300-s components, each followed by a 60-s timeout lasting approximately 70-min. Drug experiments began once rates and patterns of responding were stabilized. Stable responding was defined as no more than 20% variation of overall rates of responding for at least 3 consecutive days.

b. Cocaine self-administration

The subjects were initially trained under a second-order FI schedule of stimulus termination prior to catheter implantation in order to extend the life of the catheter. The subjects began training with a fixed ratio of one response (FR1) in the presence of a red light. Each response resulted in the red light being extinguished and a 15-s illumination of a white light followed by a 60-s time out. The ratio requirement was gradually increased as responding increased. When the ratio requirement reached FR20, a 2 s white light was presented after each completion of the FR. The FI was gradually increased and followed by a 20-s limited hold. During the limited hold, the subject had 20 s to complete the ratio requirement and extinguish the red light. Upon termination of the red light, a white light was illuminated for 15 s followed by a time out. If the red light was not extinguished during the 20 s period, the animal received a brief (200 ms) 4 mA stimulus to the tail followed by a time out. Ultimately, the schedule comprised of 5 components with an FI of 10 min, followed by a 20-s LH and a 60-s time out. When rates and patterns of responding were stabilized, venous catheters were implanted as previously described. Subsequently, the method of reinforcement was changed such that termination of the red light resulted in injection of 0.1 mg/kg of cocaine and the LH was increased to 200 s. All other events and schedule parameters remained identical to those of the second-order schedule of stimulus termination. This technique has been effective at maintaining high rates of responding during drug self-administration (Czoty et al., 2002; Ginsburg et al., 2005; Howell and Byrd, 1991; Howell et al., 1997; Howell et al., 2000; Kimmel et al., 2005; Kimmel et al., 2007; Schindler et al., 1988). Drug experiments began once rates and patterns of responding were stabilized. Initially, a range of doses of cocaine (0.03–1.0 mg/kg/injection) were substituted for the training dose of 0.1 mg/kg/injection to establish a full dose-effect curve for cocaine self-administration and to determine the dose that maintained peak rates of responding in each animal. Subsequently, subjects were maintained on the dose that maintained peak

rates of responding (0.1 mg/kg/infusion: s172, s174, s183, s184, s207, s209; 0.3 mg/kg/infusion: s179, s181, s186).

c. Reinstatement

Subjects were initially trained on a second-order schedule of stimulus termination as described above [FI 10-min(FR20:S)]. Once rates of responding were stable, subjects were implanted with an indwelling catheter as described above. The schedule parameters for the reinstatement procedures were the same as those for the self-administration procedures. A full dose-effect curve for cocaine self-administration was established to determine the dose that maintained peak rates of responding before reinstatement studies began (0.1 mg/kg/infusion: s174, s183, s184, s201, s209; 0.3 mg/kg/infusion: s186). Once rates of responding were stable at the maintenance dose for 4-5 days, subjects were then extinguished for at least two days. During extinction, the schedule parameters remained the same, except the cocaine-paired stimulus was removed and cocaine was replaced with vehicle (saline) for self-administration. Reinstatement experiments were initiated once the extinction rates were less than 30% of the cocaine-maintained response rates. During the reinstatement experiments, vehicle was available for self-administration and the cocaine-paired stimulus was restored. Studies have demonstrated that reinstatement of previously extinguished cocaine self-administration was greatest when cocaine priming was accompanied by presentations of the cocaine-paired stimulus (Spealman et al., 2004).

E. PHARMACOLOGICAL STUDIES

1. Microdialysis experiments – Nonhuman Primates

Subjects were tested once per week and each site was accessed no more frequently than once every 2 weeks. Repeated access to the caudate with the methods employed has produced consistent responses to drug treatments without significant

gliosis (Czoty et al., 2000). The probe was placed in the cannulae the morning of the experiments. Following a 60-min equilibration, four consecutive 10-min samples were collected for determination of baseline dopamine concentration. After baseline samples were collected, subjects were administered a bolus injection of saline, cocaine, or amphetamine (i.m.) and 12 samples were collected every 10 min in microcentrifuge tubes and immediately refrigerated.

Initially, the effects of manipulating the cystine-glutamate transporter on dopamine levels were examined. Three subjects (s147, s152, and s155) were administered pretreatments of NAC (3.0 or 10.0 mg/kg) systemically (i.m.) in their home cage 3 hr prior to collecting the 4 baseline samples, then cocaine (1.0 mg/kg) or saline was administered (i.m.) after the baseline samples were collected. To compare the effects of NAC on amphetamine-induced increases in dopamine, another group of four subjects (s134, s149, s156, s157), was administered pretreatments of NAC (3.0 or 10.0 mg/kg) systemically (i.m.) 3 hr prior to collecting the 4 baseline samples, then amphetamine (1.0 mg/kg) or saline was administered (i.m.) after the collection of the baseline samples.

To examine the effects of directly manipulating the mGluR2/3 receptors on dopamine levels, four subjects (s159, s168, s175, and s181) were administered LY379268 (1.0 or 3.0 mg/kg) prior to administering saline or cocaine. Following a 60-min equilibration, four consecutive 10-min samples were collected for determination of baseline dopamine concentration. Following collection of baseline samples, saline or LY379268 was administered. Three 10-min samples were collected after the pretreatment, allowing for a 30-min pretreatment before administering saline or cocaine. After cocaine or saline administration, samples were collected every 10 min for 2 hr. All samples were collected in microcentrifuge tubes and immediately refrigerated.

Systemic administration of the compounds of interest did not appear to have an effect on extracellular glutamate with the method of analysis used. Glutamate is highly regulated to prevent excitotoxicity and this regulation can prevent visualization of changes induced by drugs administered systemically. Therefore, to determine whether local drug administration would induce robust changes in extracellular glutamate, aCSF containing the cystine-glutamate enhancer, NAC, or the glutamate uptake inhibitor, THA, was administered via the analytical probe. Following a 60-min equilibration, four consecutive 10-min samples were collected for determination of baseline dopamine or glutamate concentration. Following collection of baseline samples, increasing doses of THA or NAC were infused. Each dose was infused over 30 min and 3 10-min samples were collected. NAC (0.1-0.3 μM) was administered directly into the caudate of 4 subjects (s159, s165, s175, s181) and directly in the nucleus accumbens of 4 subjects (s168, s180, s181, s188). Four subjects (s159, s165, s175, s181) were administered THA (10-30 μM) directly into the caudate. Only the lowest dose tested increased extracellular glutamate and to verify if regulatory mechanisms prevented the higher doses from inducing changes in extracellular glutamate after administering the low dose, the highest dose was administered alone for 60 min and 6 10-min samples were collected. Due to site attrition, s181 did not complete the single dose experiment.

Probes were tested in vitro to determine the suitability of probe efficiency and performance before and after each experiment. The in vitro probe recovery was stable at approximately 15-20%. However, the concentration of dopamine was not adjusted for in vitro probe recovery because techniques such as no-net flux have shown that in vitro recovery does not reflect in vivo recovery rates (Parsons and Justice, 1992). The in vivo dynamics such as release and uptake of dopamine during microdialysis greatly influence the probe recovery, leading to greater recovery in vivo as compared to in vitro. Thus, the

in vitro probe recovery was only utilized to monitor the stability of probe efficiency throughout an experiment.

2. Microdialysis experiments – Rodents

Previous studies that characterized the effect of NAC on the behavioral effects of cocaine were conducted in rodent models (Baker et al., 2003a; Madayag et al., 2007; Moran et al., 2005; Xi et al., 2002b). The current studies attempted to extend these findings into the nonhuman primate model. However, in the nonhuman primate model, systemic administration of NAC did not appear to have any effects on extracellular glutamate. There were distinct differences between previous rodent studies and the procedures utilized in these nonhuman primate studies. First, the effects of NAC on cocaine-induced behavior in rats was correlated to neurochemical changes of glutamate and dopamine in the nucleus accumbens (Baker et al., 2003a; Madayag et al., 2007; Moran et al., 2005; Xi et al., 2002b), whereas in the nonhuman primates behavior was correlated to neurochemical changes in the caudate nucleus. Second, the rodents were subjected to a sensitization paradigm of noncontingent chronic cocaine administration and withdrawal prior to the NAC administration. The nonhuman primates had an extensive cocaine self-administration history prior to the neurochemical studies, but were not subjected to a sensitization regimen. Third, in the previous rodent studies, glutamate was measured via HPLC with florescence detection, while in the nonhuman primates changes in extracellular glutamate were measured using microdialysis and HPLC with tandem mass spectrometry. In order to interpret the data from the different species and different techniques utilized, the rodent experiment was repeated using naïve rats. The rats were then subjected to chronic cocaine administration and withdrawn. They were subsequently given a NAC challenge and the changes in

extracellular glutamate in the caudate-putamen were measured using in vivo microdialysis with tandem mass spectrometry.

Initially, subjects underwent repeated daily administration of saline (N=5) or cocaine (N=7) for 7 days. Subjects that received cocaine were given 15 mg/kg s.c. on days 1 and 7 and 30 mg/kg s.c. on days 2-6. Rats then underwent a 3-week withdrawal period in which no cocaine or saline was administered. During the 3-week withdrawal period, subjects were implanted with microdialysis cannulae. During the microdialysis experiment, the subjects that received chronic saline were administered 60 mg/kg NAC s.c. after the baseline samples were collected. The subjects that received the chronic cocaine regimen were divided into 2 groups. One group (N=3) received saline s.c. during the microdialysis experiment. The second group (N=4) received 60 mg/kg NAC s.c. during the microdialysis experiment. Six 20-min samples were collected post drug administration.

3. Fixed-interval stimulus termination studies

Experimental sessions began once subjects were fully trained on the fixed-interval stimulus termination schedule and patterns and rates of responding were stable. Subjects were tested five days a week. Subjects did not receive any drug treatments on the first day of the week or on the day before or after an experimental drug was administered. The five-day schedule typically consisted of: 1. no drug session, 2. drug session, 3. no drug session, 4. vehicle session, and 5. drug session.

Initially, the effects of manipulating the cystine-glutamate transporter on the behavioral-stimulant effects of cocaine were examined. For drug time-course studies, a session consisted of 15 consecutive FI 300-s components, each followed by a 60-s time out. A cocaine dose-response curve was established for each subject by giving a single bolus dose of cocaine (0.03 -1.0 mg/kg) 5 s before the start of the session. Pretreatment

doses of NAC (3.0, 10.0, or 17.0 mg/kg) were given i.m. (volume of 0.4-0.8 mL in the thigh) in the home cage 30 min or 3 hr prior to the injection of saline, cocaine (0.3 mg/kg) or amphetamine (0.1 mg/kg) 5 s before the start of the session. In one group of subjects (s170, s171, s181, s185) with implanted indwelling i.v. catheters, NAC pretreatments were administered i.m. and cocaine was administered i.v. In a separate group of subjects (s117, s170, s171, s177), NAC and cocaine or amphetamine were administered i.m. in the thigh.

To characterize the effects of manipulating mGluR2/3 on the behavioral-stimulant effects of cocaine, four subjects (s178, s185, s187, and s190) were pretreated with LY379268 (1.0, 1.7 or 3.0 mg/kg) or LY341495 (1.0, 3.0, or 10.0 mg/kg) 5 s prior to the start of the session. Due to limited quantities of the mGluR2/3 agonist LY379268, it was given as a pretreatment to cumulative doses of cocaine. Cocaine (0.03-1.0 mg/kg) was administered via i.v. infusion (volume of 0.25 mL and flushed with 0.25 mL saline) in increasing cumulative doses after components 1, 4, 7 and 10. During control sessions, saline was administered via i.v. infusion (volume of 0.5 mL) after the same components. Before the experiments, a cumulative dose response curve was established for each subject. Pretreatment doses of the mGluR2/3 agonist, LY379268, and the mGluR2/3 antagonist, LY341495, or saline were given via i.m. injection (volume of 0.4-0.8 mL in the thigh) in the test chamber 5-min prior to the initiation of the session. Rates of responding were stable within each dose, thus the rates of responding for the 3 components following the injection were averaged to determine the rates for each dose. Each subject received each experimental combination twice, and the data for the two sessions were averaged.

4. Drug self-administration experiments

Drug experiments began once rates and patterns of responding were stable. Initially, a range of doses of cocaine (0.03–1.0 mg/injection) was substituted for the training dose to establish a full dose-effect curve for cocaine self-administration and to determine the dose that maintained peak rates of responding. Subsequently, subjects were maintained on the dose that maintained peak rates of responding (0.1 mg/kg/infusion: s183, s184, s207, s209; 0.3 mg/kg/infusion: s181, s186). Mean rate of responding during self-administration sessions was determined for individual monkeys by averaging response rate in the presence of the red light during all components of a session. Mean response rate for a given pretreatment dose was determined across 3 consecutive drug sessions and expressed as a percentage of the mean rate during the 3 days which saline was administered.

Initially, the effects of manipulating the cystine-glutamate transporter on the reinforcing effects of cocaine were examined in four subjects (s172, s174, s179, s184). During drug interaction studies, saline was administered as a control at either 30-min or 3-hr pre-session for 3 consecutive days (typically Tuesday, Wednesday, and Thursday). Subsequently, 1 dose of NAC was administered 30-min or 3-hr pre-session for 3 consecutive days of the following week. To characterize the effects of manipulating mGluR2/3 on the behavioral-stimulant effects of cocaine, five subjects (s181, s183, s186, s207, s209) were administered LY379268 pretreatments 5-min before the start of each session. To establish baseline self-administration rates, saline was administered as a control 5-min pre-session for 3 consecutive days (typically Tuesday, Wednesday, and Thursday). Subsequently, a single dose of LY379268 was administered 5-min pre-session for 3 consecutive days of the following week.

5. Reinstatement experiments

The schedule parameters for the reinstatement procedures were the same as those for the self-administration procedures. A full dose-effect curve for cocaine self-administration was established to determine the dose that maintained peak rates of responding before reinstatement studies began (0.1 mg/kg/infusion: s172, s174, s183, s184, s201, s209; 0.3 mg/kg/infusion: s179, s186). Once rates of responding were stable at the maintenance dose for 4-5 days, subjects were then extinguished for at least two days. During extinction, the schedule parameters remained the same, except the cocaine-paired stimulus was removed and cocaine was replaced with vehicle (saline) for self-administration. Reinstatement experiments were initiated once the extinction rates were less than 30% of the cocaine-maintained response rates. During the reinstatement experiments, vehicle (saline) was available for self-administration and the cocaine-paired stimulus was restored. Reinstatement probes were conducted on three consecutive days utilizing the same pretreatment drug dose. Before testing a new pretreatment dose, subjects were allowed to self-administer cocaine until baseline cocaine self-administration rates were re-established and then subjects were extinguished again. Baseline rates of responding between reinstatement probes did not vary by more than 20% and were reached within 4-5 days of cocaine self-administration. All subjects readily extinguished within 2 days of implementing extinction parameters. Mean rate of responding during reinstatement sessions was determined for individual monkeys by averaging response rate in the presence of the red light during all components of a session. The mean response rate for a given pretreatment dose and prime was expressed as a percentage of the mean rate of cocaine self-administration during the maintenance phase.

Initially, the effects of manipulating the cystine-glutamate transporter on reinstatement of previously extinguished cocaine self-administration were examined in

four subjects (s172, s174, s179, s184). Reinstatement sessions were conducted on three consecutive days utilizing the same pretreatment dose of NAC. NAC was administered 30-min or 3-hr prior to administering either an i.v. saline prime or cocaine prime (0.3 or 1.0 mg/kg) in a pseudo-randomized order. S174 lost his viable catheter sites before completing the 30-min pretreatment experiments. To characterize the effects of mGluR2/3 modulation on reinstatement of previously extinguished cocaine self-administration, four subjects (s183, s186, s201, s209) were administered LY379268 as a pretreatment during reinstatement probes. LY379268 was administered 5-min prior to administering either an i.v. saline prime or cocaine prime (0.3 or 1.0 mg/kg) in a pseudo-randomized order. The prime was administered 5 min prior to the start of the session.

IV. Results – Effects of cystine-glutamate transporter modulation on the neurochemical and behavioral effects of cocaine

A. Introduction

Interactions between dopamine and glutamate are a major focus in the areas of drug addiction and mental health. Anatomical substrates for these interactions have been established in rodents (Sesack et al., 2003). Though dopamine signaling has functions independent of glutamate, glutamate regulates some of the critical modulatory functions of dopamine. Glutamatergic afferents from the prefrontal cortex and the laterodorsal/pedunculopontine tegmentum innervate dopamine neurons. The regulation by these neurons is critical for phasic firing of dopamine to signal behavioral events (Sesack et al., 2003). Midbrain dopamine neurons also project to the cerebral cortex and synapse on both glutamatergic pyramidal cells and GABAergic medium spiny neurons and interneurons in both rodents and nonhuman primates. Understanding this reciprocal signaling may be important for developing pharmacotherapies of cocaine addiction.

The glutamate system has been linked to locomotor sensitization, conditioned place preference, and drug self-administration (Carlezon and Nestler, 2002; Cornish and Kalivas, 2000, 2001; Pierce et al., 1996), all of which are behavioral measures often used to model drug abuse. Acute administration of cocaine can increase synaptic release of both dopamine and glutamate in the nucleus accumbens (McFarland et al., 2003). In contrast, repeated cocaine administration decreases basal extrasynaptic glutamate (Baker et al., 2002a). Although vesicular release of glutamate contributes to the basal tone of glutamate extracellular, glutamate seems to originate primarily from cystine/glutamate exchange via transporters located predominantly on glial cells (Baker et al., 2002b; Pow, 2001). This extrasynaptic glutamate provides tonic regulation on

dopamine function in the mesocorticolimbic pathway via group II metabotropic receptors (mGluR2/3), which act as autoreceptors to regulate pre-synaptic neurotransmitter release (Baker et al., 2003a; Baker et al., 2002b). Repeated cocaine administration decreases basal glutamate in rodents (McFarland et al., 2003), which results in decreased activation of mGlu2/3 receptors, which in turn facilitates synaptic release of glutamate and dopamine. In rodents, restoring basal glutamate levels by enhancing cystine-glutamate exchange blocks reinstatement of previously extinguished cocaine self-administration (Baker et al., 2003a). Understanding the mechanisms by which glutamate modulates dopamine in the context of cocaine neuropharmacology may uncover new targets in developing pharmacotherapeutic medications for cocaine addiction.

B. Results

1. Microdialysis

Cocaine administration (i.m.) produced a dose-dependent increase in extracellular dopamine as measured by in vivo microdialysis ($F[30,143]=6.156$, $p<0.001$; Figure 1A). In subsequent experiments, when a 3-hr pretreatment of NAC was given before a saline injection, there was not a significant change in extracellular dopamine ($F[30,95]=0.524$, $p=0.959$; Figure 1B). However, a 3-hr pretreatment of NAC significantly attenuated cocaine-induced increases in dopamine ($F[45,191]=3.159$, $p=<0.001$; Figure 1C). Though not significant, pretreatment with a low dose of NAC tended to enhance amphetamine-induced increases in dopamine, whereas a higher dose of NAC tended to attenuate amphetamine-induced increases in dopamine (Figure 1D). Accordingly, NAC had differential effects on cocaine and amphetamine-induced increases in extracellular dopamine. However, it is important to note that cocaine produced a 300 % increase in dopamine, while amphetamine produced a 750% increase

in dopamine. Thus, the differential effects of NAC may be due to the differences in potency between the psychostimulants. Further studies in which the doses of the psychostimulants are matched for potency are needed.

A high level of extracellular glutamate is highly excitotoxic. Thus, extracellular glutamate is tightly regulated to prevent neurotoxicity. This regulation can prevent visualization of changes induced by drugs administered systemically in methods such as microdialysis that are limited by time resolution and size of the membrane probe (Drew et al., 2004). In the current studies, systemic administration of the compounds of interest did not appear to have an effect on extracellular glutamate. Therefore, to determine whether local drug administration would induce robust changes in extracellular glutamate, aCSF containing the cystine-glutamate enhancer, NAC, or the glutamate uptake inhibitor, THA, was administered via the analytical probe. Following collection of baseline samples, increasing doses of NAC (0.03-0.3uM) or THA (30-300uM) were infused. Each dose was infused over 30-min. In the current studies, NAC was administered systemically under the assumption that it was enhancing extracellular glutamate via the cystine-glutamate transporter. Initially, NAC was infused directly into the caudate nucleus in order to determine if NAC was enhancing cystine-glutamate transport and extracellular glutamate. Increasing doses of NAC did not significantly alter extracellular glutamate ($F[14,57]=0.845$, $p=0.619$; Figure 2A). However, there appeared to be a trend towards an increase with the lowest dose that quickly recovered to baseline levels. In the rodent studies, changes in glutamate in response to NAC were observed in the nucleus accumbens; therefore NAC was administered directly into the nucleus accumbens. Direct administration of NAC into the nucleus accumbens did not significantly alter extracellular glutamate (Chi-square[11]=5.133, $p=0.925$, Figure 2B). However, there was a tendency for the lower two doses of NAC to increase extracellular glutamate and rapidly recover to baseline levels. There appeared to be a distinct

difference between how glutamate levels were regulated in the nucleus accumbens versus the caudate nucleus. There appears to be a tighter regulation of extracellular glutamate in the caudate. It is well established that there is a higher density of dopamine transporters in the caudate nucleus than in the nucleus accumbens. This may also be true for other transporters such as the excitatory amino acid transporters. A higher density of these transporters would result in a more rapid reuptake of glutamate and quicker regulation of extracellular glutamate that would prevent the visualization of dramatic changes in extracellular glutamate with a sampling time of 10-min. Rapid sampling techniques such as voltammetry or enzyme-immobilized biosensors that can measure real-time changes in neurotransmitters may be better suited to characterize changes in extracellular glutamate in the nonhuman primate model.

The effects of NAC on extracellular glutamate may not have been robust enough to be visualized in microdialysis. Consequently, the glutamate reuptake inhibitor, THA, was infused in order to determine if blocking reuptake would induce robust changes in extracellular glutamate. Direct infusion of THA did not significantly alter extracellular glutamate when infused with increasing doses ($F[13,54]=1.037$, $p=0.439$; Figure 3A), though there appeared to be a trend towards an increase with the lowest dose that quickly recovered to baseline levels. The lower dose of THA may not have been sufficient enough to overcome the mechanisms that regulate extracellular glutamate, which prevented a significant increase in extracellular glutamate. Moreover, the initial dose of THA may have triggered regulatory mechanisms that prevented subsequent increases in extracellular glutamate with increasing doses of THA. Thus, the highest dose was administered alone over a 1-hr period to determine if the higher dose would significantly increase extracellular glutamate. There was a significant increase in extracellular glutamate when the high dose of THA was administered alone ($F[8,16]=2.774$, $p=0.039$; Figure 3B). Hence, the mechanisms that regulate extracellular glutamate must be

overcome in order to visualize significant changes in extracellular glutamate with microdialysis in the caudate of squirrel monkeys.

The current studies attempted to extend previous findings that characterized the effect of NAC on the behavioral effects of cocaine in rodent models (Baker et al., 2003a; Madayag et al., 2007; Moran et al., 2005; Xi et al., 2002b) into the nonhuman primate model. Though systemic administration of NAC in the nonhuman primate model attenuated extracellular dopamine as would be expected from previous rodent studies, NAC did not appear to have any effects on extracellular glutamate or on the behavioral pharmacology of cocaine in the nonhuman primate (discussed in next results sections). In order to interpret the data from the different species and different techniques utilized, the rodent experiment was repeated using naïve rats. The rats were then subjected to chronic cocaine administration and withdrawn. They were subsequently given a NAC challenge and the changes in extracellular glutamate in the caudate-putamen were measured using in vivo microdialysis with tandem mass spectrometry. There were three treatment groups: 1. Chronic saline treatment with NAC challenge (SAL-NAC), 2. Chronic cocaine treatment with saline challenge (COC-SAL) and 3. Chronic cocaine treatment with NAC challenge (COC-NAC). There was a significant main effect of treatment between treatment groups ($F[2,160]=13.907$, $p<0.001$; Figure 4). Post hoc analysis revealed no significant difference between the SAL-NAC and COC-SAL groups; however, there was a significant increase in extracellular glutamate in the COC-NAC group as compared to the other treatment groups. This is consistent with previous rodent studies that report that NAC only induced an increase in extracellular glutamate in the nucleus accumbens after glutamate is dysregulated by chronic cocaine treatment. Thus, the lack of effect of NAC on extracellular glutamate and on cocaine-induced behavioral effects in nonhuman primates is most likely due to a species difference rather than differences in brain regions examined or techniques used.

2. Fixed-interval stimulus termination.

In the first set of behavioral experiments, NAC was given as a pretreatment in subjects trained to respond under a fixed-interval schedule of stimulus termination. Stable rates of responding were maintained throughout the 90-min session following i.v. administration of saline, and NAC pretreatments had no behavioral effects (30-min: $F[42,239]=1.332$, $p=0.114$; 3-hr: $F[42,239]=1.110$, $p=0.324$.; Figure 5A,C). However, after i.v. administration of cocaine, rates of responding significantly increased within the first 20 minutes of the session and returned to baseline levels by 60 minutes (30-min: $F[14,239]=9.207$, $p<0.001$; 3-hr: $F[14,239]=9.355$, $p=<0.001$; Figure 5B,D). Pretreatment with NAC did not significantly alter cocaine-induced changes in response rates (30-min: $F[42,239]=1.394$, $p=0.082$; 3-hr: $F[42,239]=1.081$, $p=0.363$). These results did not correlate with changes observed in neurochemistry. In a different group of subjects, NAC pretreatments and cocaine or saline were administered i.m. Saline administered i.m. had no systematic effect on responding throughout the behavioral session, and NAC pretreatments had no behavioral effects alone (30-min: $F[28,179]= 2.237$, $p=0.003$; 3-hr: $F[28,179]=0.900$, $p=0.631$; Figure 6A,C). Though there was a significant interaction between dose and time when NAC was administered as a 30-min pretreatment before saline, posthoc analysis revealed no significant differences between the pretreatment groups. Cocaine administered i.m. significantly increased rates of responding within the first 20 minutes of the session and returned to baseline by 60 minutes (30-min: $F[14,179]= 5.589$, $p<0.001$; 3-hr: $F[14,179]=9.337$, $p=<0.001$; Figure 6B,D). Neither 30-min nor 3-hr NAC pretreatments significantly altered cocaine-induced increases in rates of responding (30-min: $F[28,179]= 0.900$, $p=0.613$; 3-hr: $F[28,179]=1.155$, $p=0.301$; Figure 6B,D). Because NAC pretreatments had differential effects on cocaine and amphetamine neurochemistry, the same four subjects repeated these experiments and were administered saline or amphetamine i.m. NAC did not alter

rates of responding when administered as a pretreatment to saline administration (30-min: $F[28,179]= 0.489$, $p=0.982$; 3-hr: $F[28,179]=1.003$, $p=0.475$; Figure 7A,C). Amphetamine administration increased rates of responding within the first 20 minutes of the session and returned to baseline within 60-min (30-min: $F[14,179]= 5.304$, $p<0.001$; 3-hr: $F[14,179]=3.841$, $p=<0.001$). Furthermore, NAC pretreatments did not alter amphetamine-induced increases in rates of responding (30-min: $F[28,179]= 1.761$, $p=0.025$; 3-hr: $F[28,179]=1.003$, $p=0.475$; Figure 7B,D). Though there was a significant interaction between dose and time when NAC was administered as a 30-min pretreatment before amphetamine, posthoc analysis revealed no significant differences between the pretreatment groups.

3. Self-administration and Reinstatement.

To determine if the attenuation of cocaine-induced increases in extracellular dopamine by NAC would result in changes in the reinforcing properties of cocaine, four squirrel monkeys trained on a second-order schedule of cocaine self-administration were given 30-min or 3-hr pretreatments of NAC prior to the behavioral sessions. During drug interaction studies, baseline responding was determined by administering saline as a pretreatment for 3 consecutive days and mean rate of responding was averaged across the sessions. Pretreatments of NAC did not significantly alter rates of responding during cocaine self-administration (30-min: $F[3,15]=4.136$, $p=0.042$; 3-hr: $F[3,15]=1.948$, $p=0.192$; Figure 8). Though there was a significant interaction between dose and time when NAC was administered as a 30-min pretreatment before cocaine self-administration, posthoc analysis revealed no significant differences between the pretreatment groups. When saline was substituted for cocaine, subjects extinguished responding within two sessions. Non-contingent injections of cocaine (i.v.) produced a dose-dependent reinstatement of previously extinguished self-administration (30-min:

F[3,11]=371.025, p=<0.001; 3-hr: F[3,15]=195.857, p=<0.001; Figure 9). However, NAC pretreatments did not significantly alter cocaine-induced reinstatement (30-min: F[6,35]=1.185, p=0.377, 3-hr: F[6, 47]=0.971, p=0.472).

C. Discussion

Dysregulation of basal glutamatergic tone has been attributed as one of the underlying mechanisms for cocaine relapse and addiction. The origin of basal extracellular glutamate is primarily from the cystine-glutamate transporter localized on astrocytes (Baker et al., 2002b). Basal glutamate provides glutamatergic tone on extrasynaptic glutamate receptors that regulate synaptic release of glutamate such as the mGluR2/3 (Xi et al., 2002a). Acute administration of cocaine in rodents increases extracellular glutamate, potentially due to increased vesicular glutamate release. However, chronic cocaine administration leads to dysregulation of glutamate and decreased basal tone (Baker et al., 2002a). In rodents undergoing chronic cocaine administration and withdrawal, the acute effects of cocaine on extracellular glutamate are enhanced, suggesting that the basal glutamatergic tone on mGluR2/3 may be an important modulator in the behavioral pharmacology of cocaine.

The purpose of the current study was to determine the effect of manipulating the cystine-glutamate transporter on cocaine-induced changes in dopamine neurochemistry in conscious nonhuman primates. Furthermore, the current studies aimed to determine if changes in dopamine neurochemistry would be reflected in the behavioral pharmacology of cocaine. Administration of the cystine prodrug, NAC, significantly attenuated cocaine-induced increases in extracellular dopamine without directly altering basal extracellular dopamine. However, this attenuation did not translate to changes in the behavioral pharmacology of cocaine. NAC pretreatment did not completely abolish cocaine-induced increases in dopamine. Interestingly, the attenuation of extracellular

dopamine by NAC observed in the current studies decreased the effects of 1.0 mg/kg cocaine to levels that were equivalent to the effects of 0.3 mg/kg cocaine. It is widely accepted that 0.3 mg/kg cocaine is a dose that is highly reinforcing and induces behavioral-stimulant effects in nonhuman primates. The current behavioral experiments utilized 0.3 mg/kg dose of cocaine to induce stimulant effects and either 0.1 or 0.3 mg/kg/inf for self-administration. Thus, a more significant attenuation of cocaine-induced increases in extracellular dopamine may be necessary for changes in behavior to be expressed. Higher doses or chronic administration of NAC may be needed to reduce dopamine sufficiently enough to see behavioral changes.

In rodents, increasing levels of cystine in the nucleus accumbens did not modify the transporter unless basal glutamate levels were altered by chronic cocaine administration (Baker et al., 2002a). In the current studies, all the subjects were cocaine experienced prior to drug interaction studies, thus it was hypothesized that direct administration of NAC would increase extracellular glutamate. Direct administration of NAC did not significantly alter extracellular glutamate, though there was a trend toward an increase with the lowest dose. Moreover, the effects of the reuptake inhibitor THA were similar to the effects of NAC. However, a high dose of THA significantly elevated extracellular glutamate. These results indicate that the mechanisms of regulating glutamate have to be overcome in order to visualize changes in extracellular glutamate using microdialysis. Furthermore, the current rodent studies were consistent with previously reported rodent studies in that NAC only increased extracellular glutamate in rodents that had received chronic cocaine treatment and were withdrawn. These results indicate that the differential effects of NAC in the rodent and nonhuman primate models were due to a species difference and not to a difference in neuroanatomical target. The caudate nucleus was targeted in the current studies because of the higher density of dopamine transporters expressed, the size of the target, and the potential of targeting

both the caudate and accumbens from the same cannulae. Most importantly, the interspersed connections between the dorsal and ventral striatum and the prefrontal cortex suggests that in nonhuman primates the two nuclei may not have as distinct roles as in the rodent brain (Haber et al., 2000; Kimmel et al., 2005; Lynd-Balta and Haber, 1994a, b). Previous studies demonstrate that cocaine-induced similar increases in dopamine in the ventromedial striatum versus the dorsalateral striatum (Bradberry, 2000) and cocaine-induced elevations in dopamine in the caudate nucleus can be modulated by environmental context cues associated with self-administration (Kimmel et al., 2005). These results further support the need to investigate pharmacotherapeutic targets in the nonhuman primate model.

The effects of NAC on amphetamine-induced increases in dopamine were inconsistent and not statistically significant. The lower dose tended to enhance the effects of amphetamine, whereas the higher dose of NAC attenuated the effects of amphetamine, as observed with cocaine. NAC ostensibly increased extrasynaptic glutamate via actions at the cystine-glutamate transporter, which results in increased signaling on mGluR2/3 receptors and decreased vesicular release of glutamate. This decreased vesicular release of glutamate would lead to decreased excitation of dopamine neurons. Given that the effects of cocaine on extracellular dopamine are impulse-dependent, reduced presynaptic tone should render cocaine less effective. In contrast, the effects of amphetamine on extracellular dopamine are impulse-independent and less likely to be influenced by presynaptic tone. Thus, the indirect effects of NAC on the excitation of dopamine neurons may explain the differential effects observed with cocaine and amphetamine.

The current study provides the first evidence that NAC can attenuate cocaine-induced increases in striatal dopamine in vivo in nonhuman primates. However, in contrast to previous studies in rodents, NAC had no significant effect on the behavioral

pharmacology of cocaine. The behavioral-stimulant, reinforcing and reinstatement effects of cocaine were not influenced by NAC administration even when dose, route of administration and pretreatment time were varied systematically. The generality of effects reported in rodents do not extend to nonhuman primates under the conditions employed.

D. Figures for the effects of NAC on the neurochemical and behavioral effects of cocaine

Figure 1 – Effects of NAC on dopamine neurochemistry

Cocaine administration (i.m.) dose-dependently increased extracellular dopamine in the caudate (A) in three squirrel monkeys. The peak response was achieved within 10-min and returned to baseline within 60-min. NAC administration (i.m.) did not significantly alter extracellular dopamine when administered as a pretreatment 3 hr prior to the administration of saline (B). However, NAC significantly attenuated cocaine-induced increases in extracellular dopamine (C). A low dose of NAC tended to enhance amphetamine-induced increases in extracellular dopamine, while a higher dose of NAC slightly attenuated amphetamine-induced increases in extracellular dopamine (D). The 10-min sampling intervals indicated began 60-min post probe insertion. Arrows indicate the point at which saline or drug was administered. Data points represent mean±SEM dopamine levels as a percent of values obtained prior to drug administration. Abscissa: time. Ordinate: extracellular concentrations of dopamine expressed as a percent of baseline control levels.

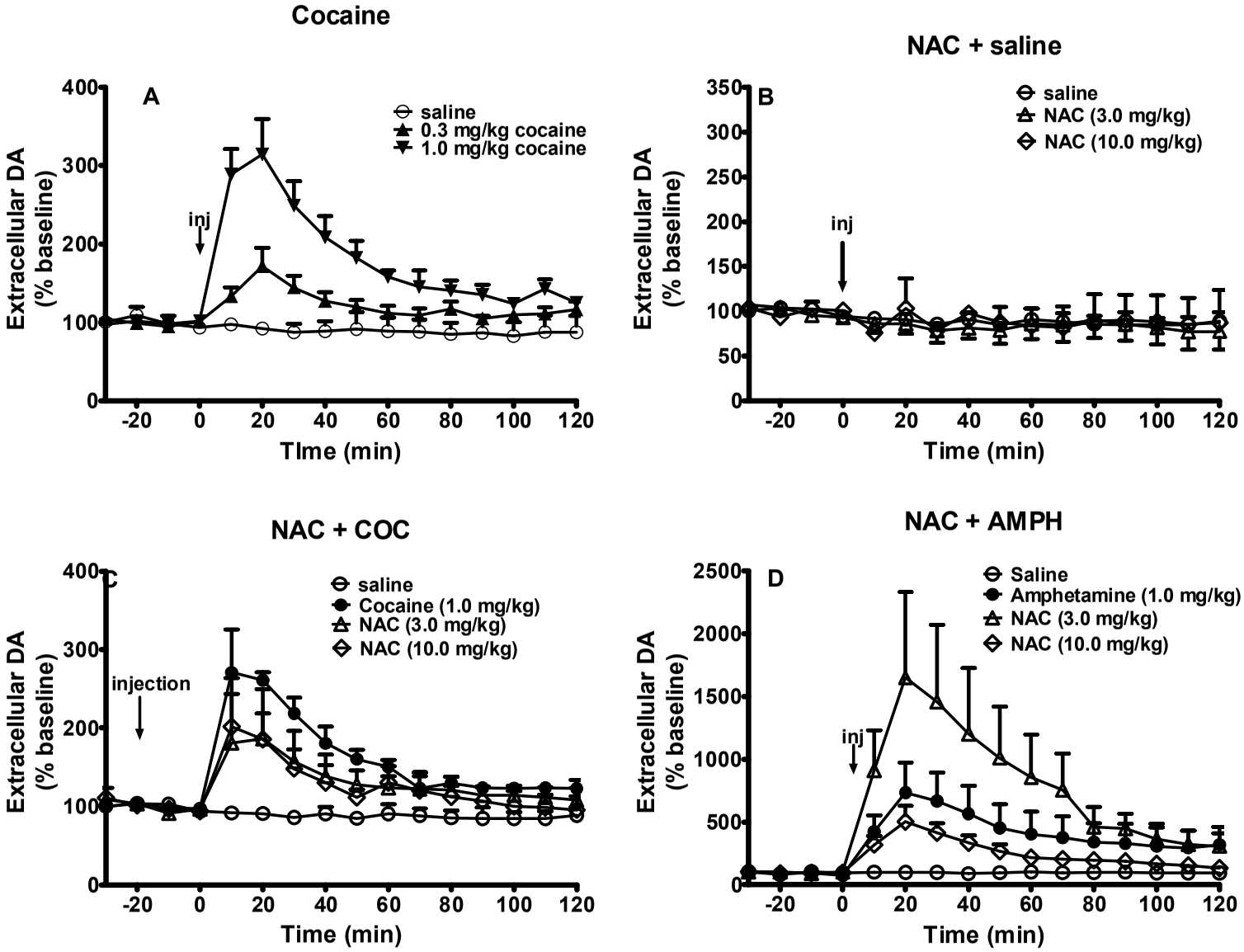
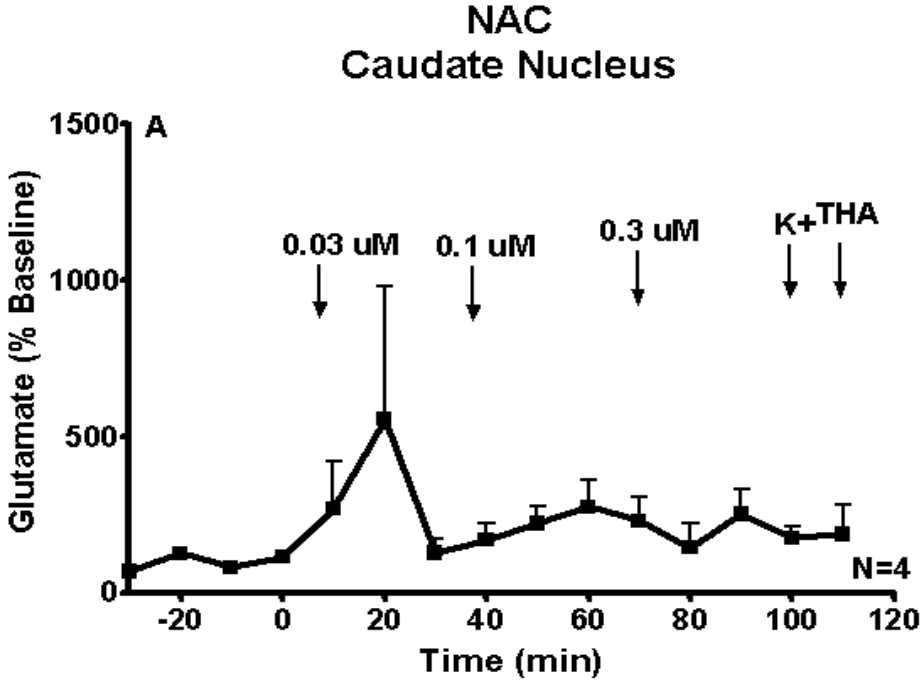

Cocaine
A
saline
0.3 mg/kg cocaine
1.0 mg/kg cocaine
inj
Extracellular DA
(% baseline)
TIme (min)
NAC + saline
B
saline
NAC (3.0 mg/kg)
NAC (10.0 mg/kg)
inj
Extracellular DA
(% baseline)
Time (min)
NAC + COC
C
saline
Cocaine (1.0 mg/kg)
NAC (3.0 mg/kg)
NAC (10.0 mg/kg)
injection
Extracellular DA
(% baseline)
Time (min)
NAC + AMPH
D
Saline
Amphetamine (1.0 mg/kg)
NAC (3.0 mg/kg)
NAC (10.0 mg/kg)
inj
Extracellular DA
(% baseline)
Time (min)

Figure 2 – Effect of direct NAC administration on glutamate neurochemistry

Direct administration of NAC in the caudate nucleus induced an increase in extracellular glutamate with the lowest dose administered but increasing doses had no affect on extracellular glutamate. Further, direct administration of NAC in the nucleus accumbens produced an increase in extracellular glutamate with all the doses tested though there was not a dose dependent effect. The 10-min sampling intervals indicated began 60-min post probe insertion. Arrows indicate the start point at which each drug dose was infused. Data points represent mean±SEM glutamate levels as a percent of values obtained prior to drug administration. Abscissa: time. Ordinate: extracellular concentrations of glutamate expressed as a percent of baseline control levels


NAC
Caudate Nucleus
A
1500
1000
500
0
Glutamate (% Baseline)
0.03 uM
0.1 uM
0.3 uM
K+
THA
N=4
-20
0
20
40
60
80
100
120
Time (min)

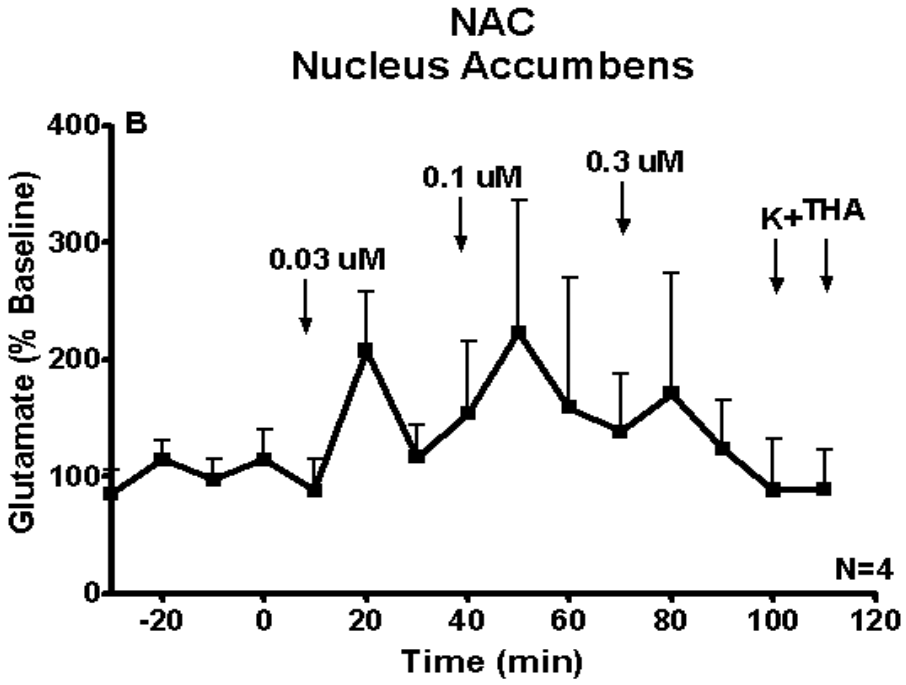
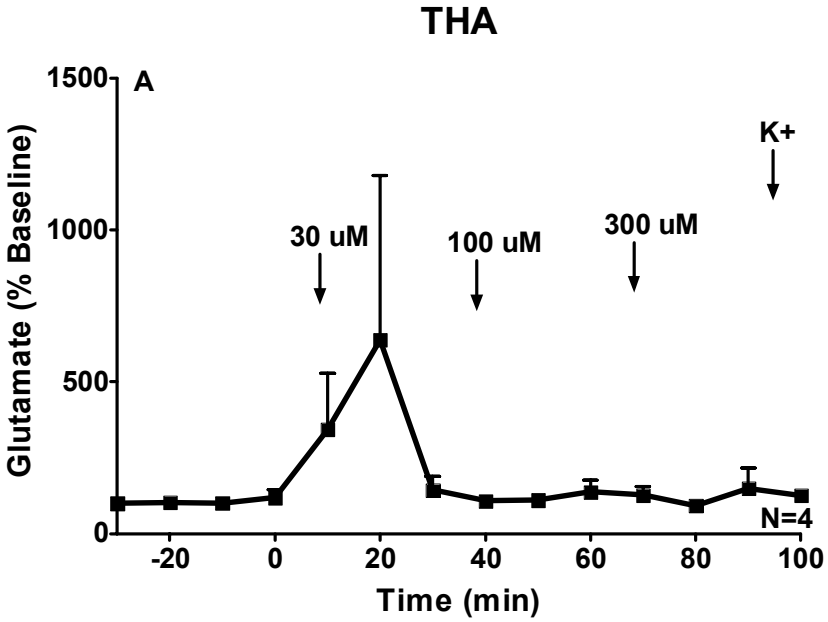

NAC
Nucleus Accumbens
B
400
300
200
100
0
Glutamate (% Baseline)
0.03 uM
0.1 uM
0.3 uM
K+
THA
N=4
-20
0
20
40
60
80
100
120
Time (min)

Figure 3 – Effect of direct THA administration on glutamate neurochemistry
(A) Direct administration of THA in the caudate nucleus tended to increase extracellular glutamate with the lowest dose administered but increasing doses had no affect on extracellular glutamate. To determine if the dose or glutamate regulation were factors in preventing a significant increase in glutamate, a highest dose of THA was infused alone for 1-hr. (B) There was a significant increase in extracellular glutamate when the high dose of THA was administered alone. The 10-min sampling intervals indicated began 60-min post probe insertion. Arrows indicate the start point at which each drug dose was infused. Data points represent mean±SEM glutamate levels as a percent of values obtained prior to drug administration. Abscissa: time. Ordinate: extracellular concentrations of glutamate expressed as a percent of baseline control levels.


THA
A
1500
1000
500
0
Glutamate (% Baseline)
30 uM
100 uM
300 uM
K+
N=4
-20
0
20
40
60
80
100
Time (min)

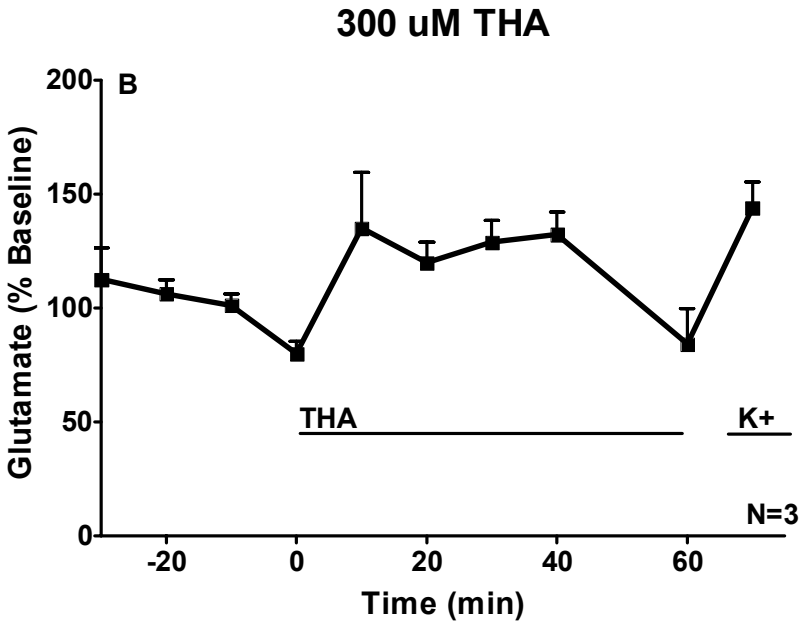

300 uM THA
B
200
150
100
50
0
Glutamate (% Baseline)
THA
K+
N=3
-20
0
20
40
60
Time (min)

Figure 4 - Effect of NAC on glutamate neurochemistry in rats after chronic cocaine or saline treatment and withdrawal.

Rodents were chronically treated for seven days with saline or cocaine (days 1,7 – 15 mg/kg, days 2-6 – 30 mg/kg). The subjects then underwent a 3-week withdrawal period. After the withdrawal period, subjects underwent microdialysis experiments in which they were given either saline or NAC (60 mg/kg). There was no significant difference between subjects receiving chronic saline treatment and NAC during the microdialysis and those receiving chronic cocaine treatment and saline during the microdialysis. However, there was a significant increase in extracellular glutamate in subjects receiving chronic cocaine treatment and NAC during the microdialysis.

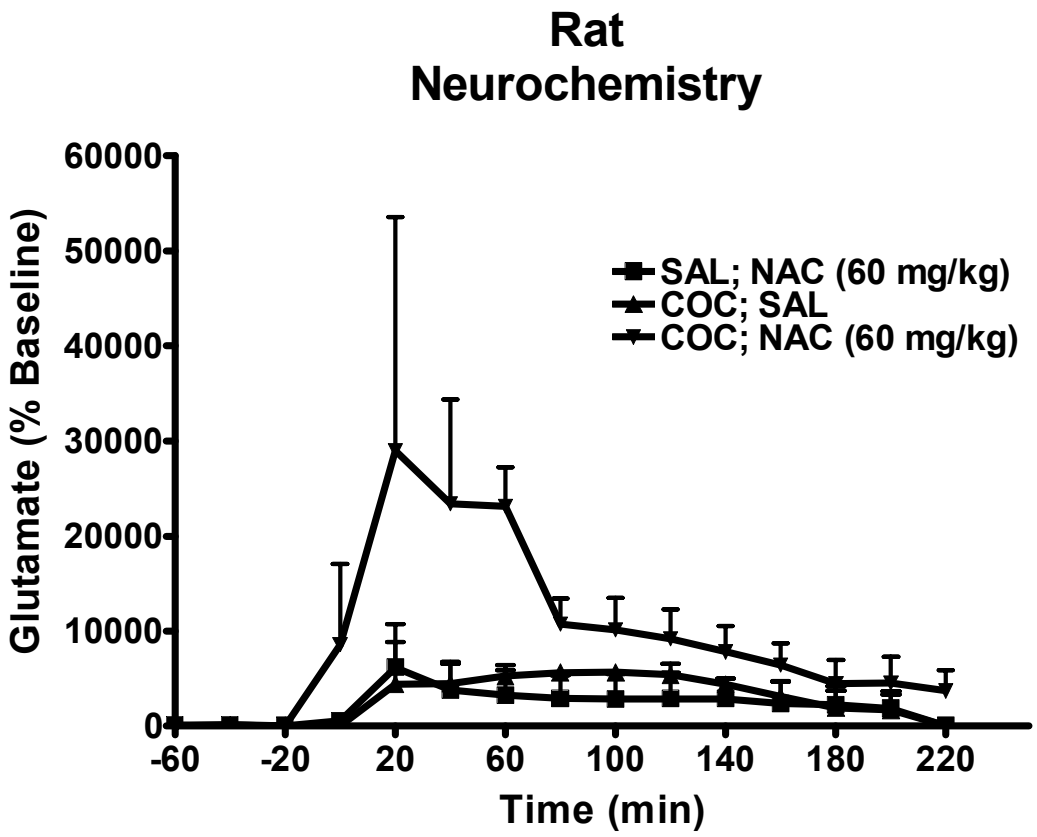

Rat
Neurochemistry
Glutamate (% Baseline)
60000
50000
40000
30000
20000
10000
0
-60
-20
20
60
100
140
180
220
Time (min)
SAL; NAC (60 mg/kg)
COC; SAL
COC; NAC (60 mg/kg)

Figure 5 – Effects of NAC on behavioral-stimulant effects of i.v. cocaine administration NAC pretreatments (i.m.) did not significantly alter rates of responding under a fixed-interval avoidance schedule when saline was administered i.v. 5-s prior to the start of the session (A, C). Cocaine significantly increased rates of responding, reaching peak rates of responding within 20-min of the start of the session. Rates of responding returned to baseline within an hour of the start of the session. However, NAC pretreatments did not significantly alter cocaine-induced increases in rates of responding when cocaine was administered i.v. 5-s prior to the session (B, D). Abscissae: drug dose or saline infusion. Ordinates: rates of responding expressed as a percent of baseline control rates.

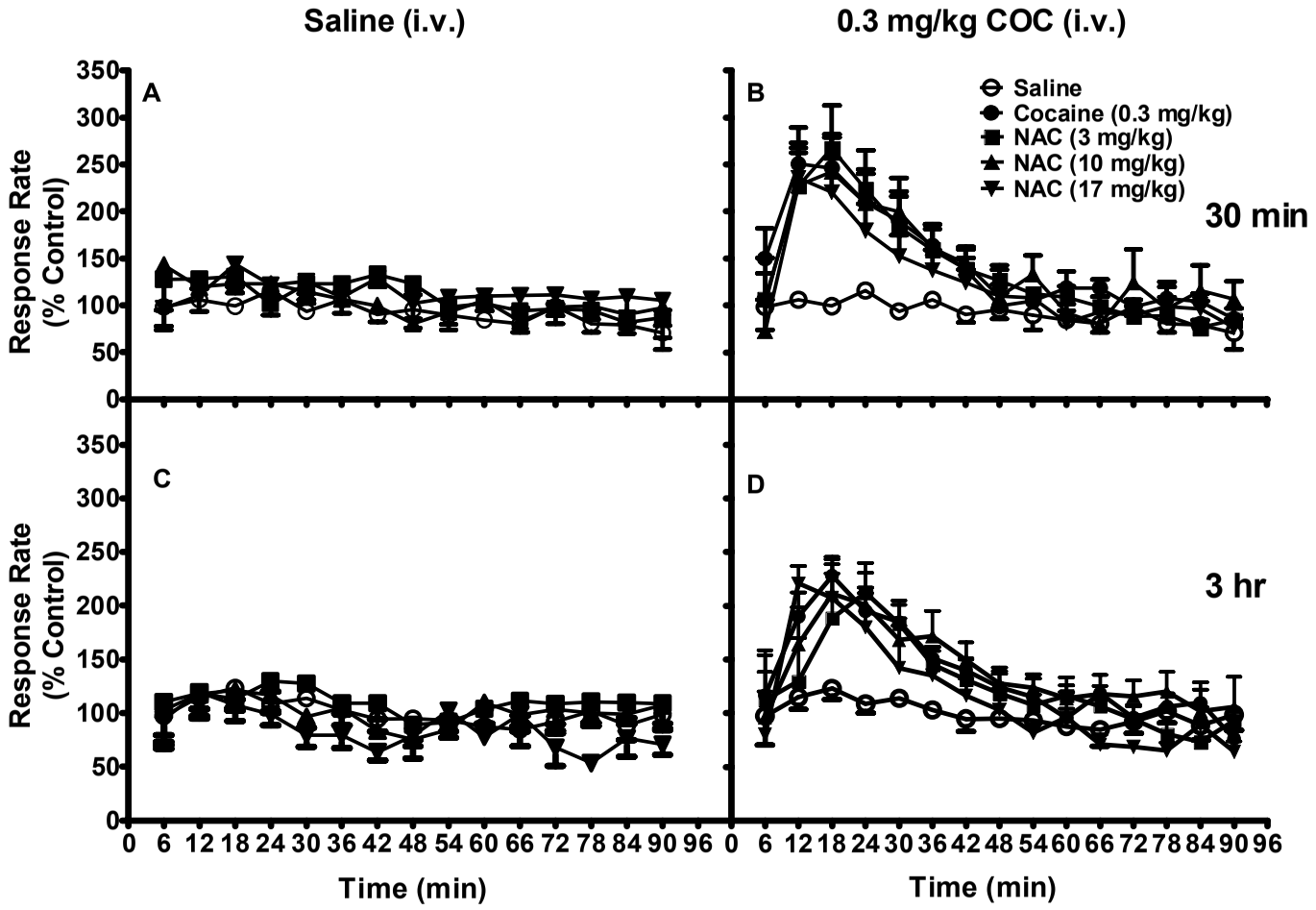

Saline (i.v.)
0.3 mg/kg COC (i.v.)
A
B
C
D
Saline
Cocaine (0.3 mg/kg)
NAC (3 mg/kg)
NAC (10 mg/kg)
NAC (17 mg/kg)
30 min
3 hr
Response Rate (% Control)
Response Rate (% Control)
0 50 100 150 200 250 300 350
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96
Time (min)
Time (min)

Figure 6 – Effects of NAC on behavioral-stimulant effects of i.m. cocaine administration NAC pretreatments (i.m.) did not significantly alter rates of responding under a fixed-interval avoidance schedule when saline was administered i.m. 5-s prior to the start of the session (A, C). Further, NAC pretreatments did not significantly alter cocaine-induced increases in rates of responding when cocaine was administered i.m. 5-s prior to the session (B,D). Abscissae: drug dose or saline infusion. Ordinates: rates of responding expressed as a percent of baseline control rates.

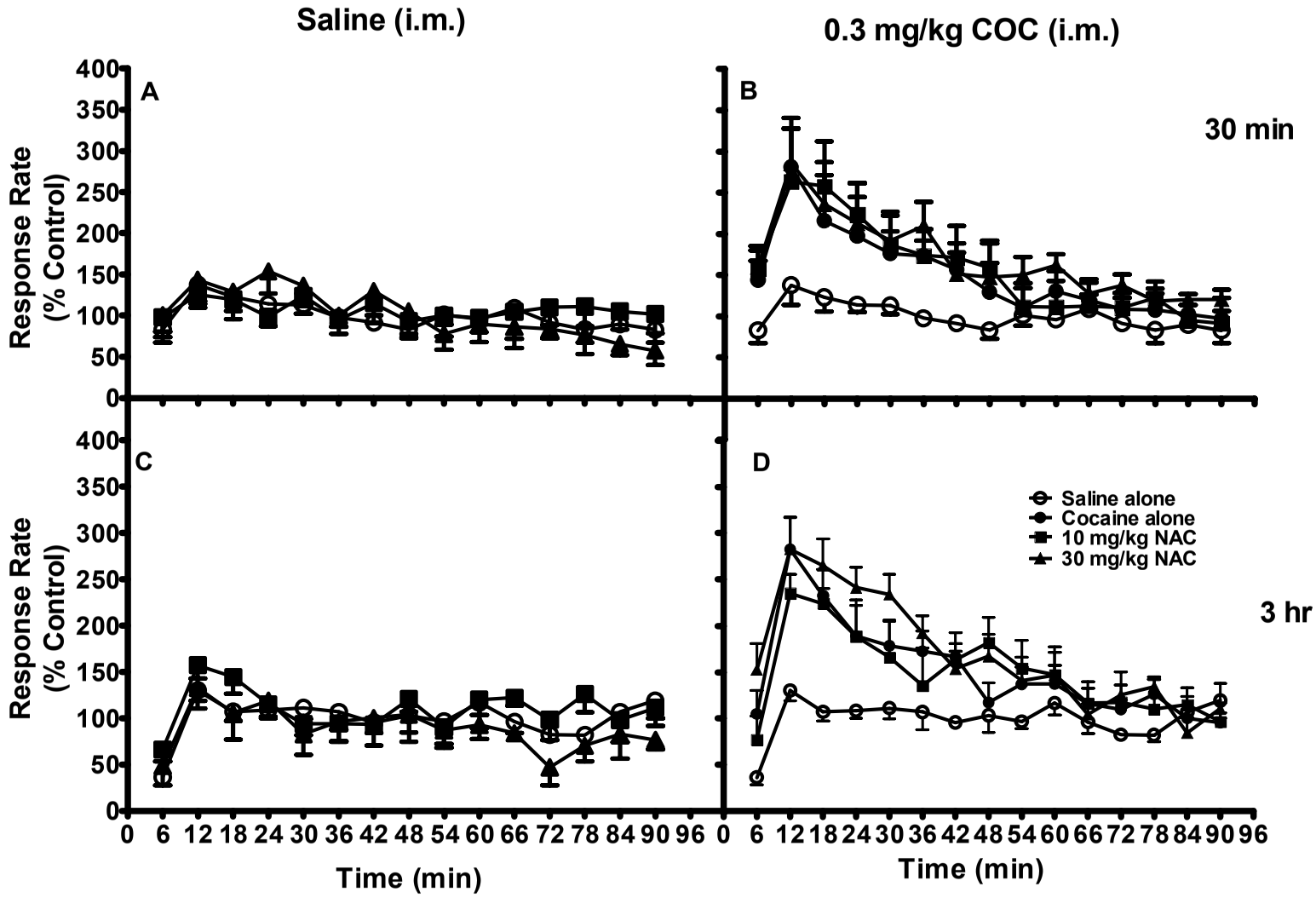

Saline (i.m.)
0.3 mg/kg COC (i.m.)
A
B
C
D
30 min
3 hr
Response Rate
(% Control)
Time (min)
Saline alone
Cocaine alone
10 mg/kg NAC
30 mg/kg NAC
0 50 100 150 200 250 300 350 400
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96

Figure 7 - Effects of NAC on behavioral-stimulant effects of i.m. amphetamine administration

NAC pretreatments (i.m.) did not significantly alter rates of responding under a fixed-interval avoidance schedule when saline was administered i.m. 5-s prior to the session (A, C). Furthermore, NAC pretreatments did not significantly alter amphetamine-induced increases in rates of responding when amphetamine was administered i.m. 5-s prior to the session (B,D).

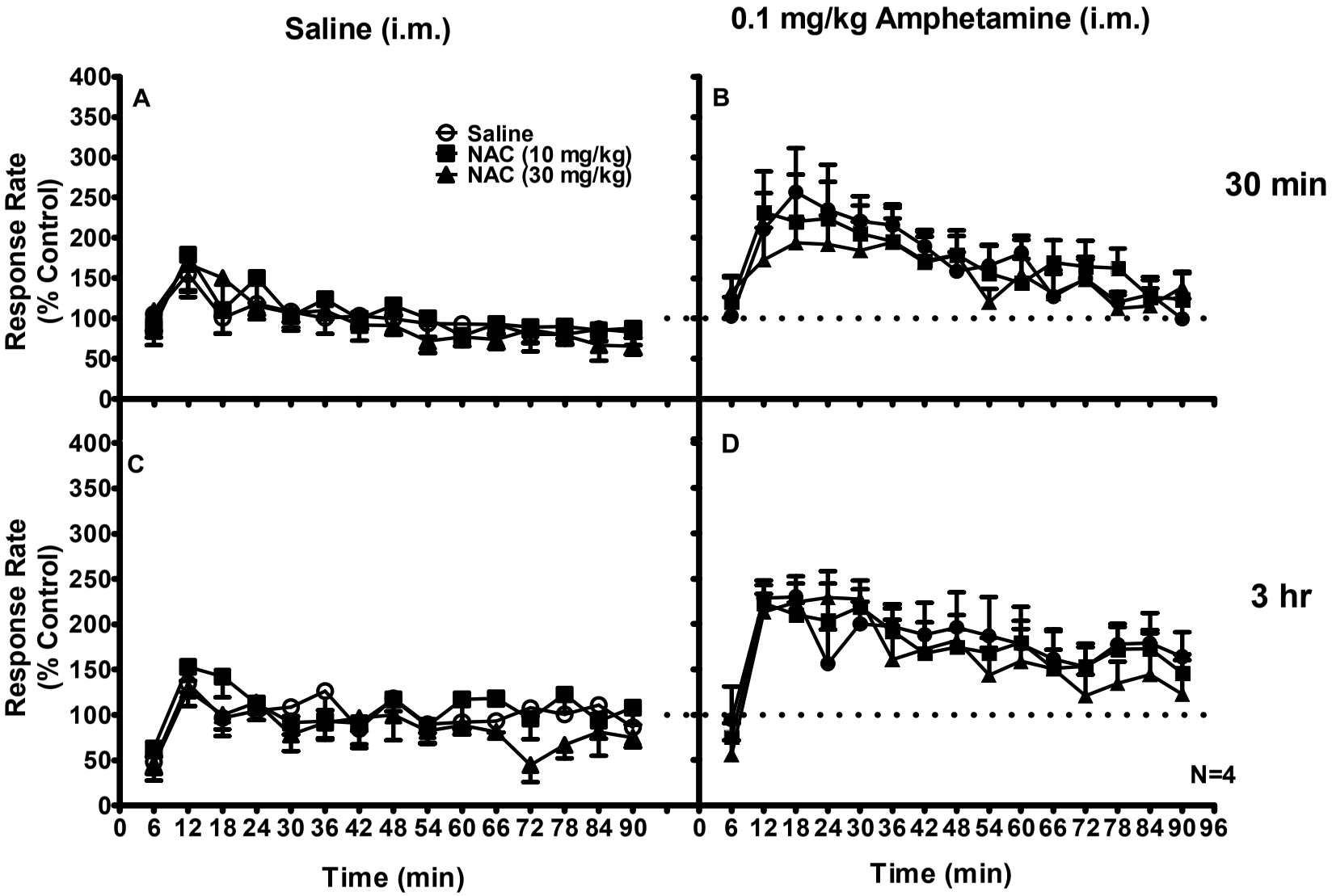

Saline (i.m.)
0.1 mg/kg Amphetamine (i.m.)
A
B
C
D
Saline
NAC (10 mg/kg)
NAC (30 mg/kg)
Response Rate (% Control)
Response Rate (% Control)
30 min
3 hr
N=4
Time (min)
Time (min)
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96
0 50 100 150 200 250 300 350 400

Figure 8 – Effects of NAC on cocaine self-administration

Administration of NAC did not significantly alter rates of responding maintained by a second-order schedule of cocaine self-administration. Each dose of NAC was administered for three consecutive days on separate occasions. Abscissa: drug dose. Ordinate: rates of responding expressed as a percent of baseline control rates.

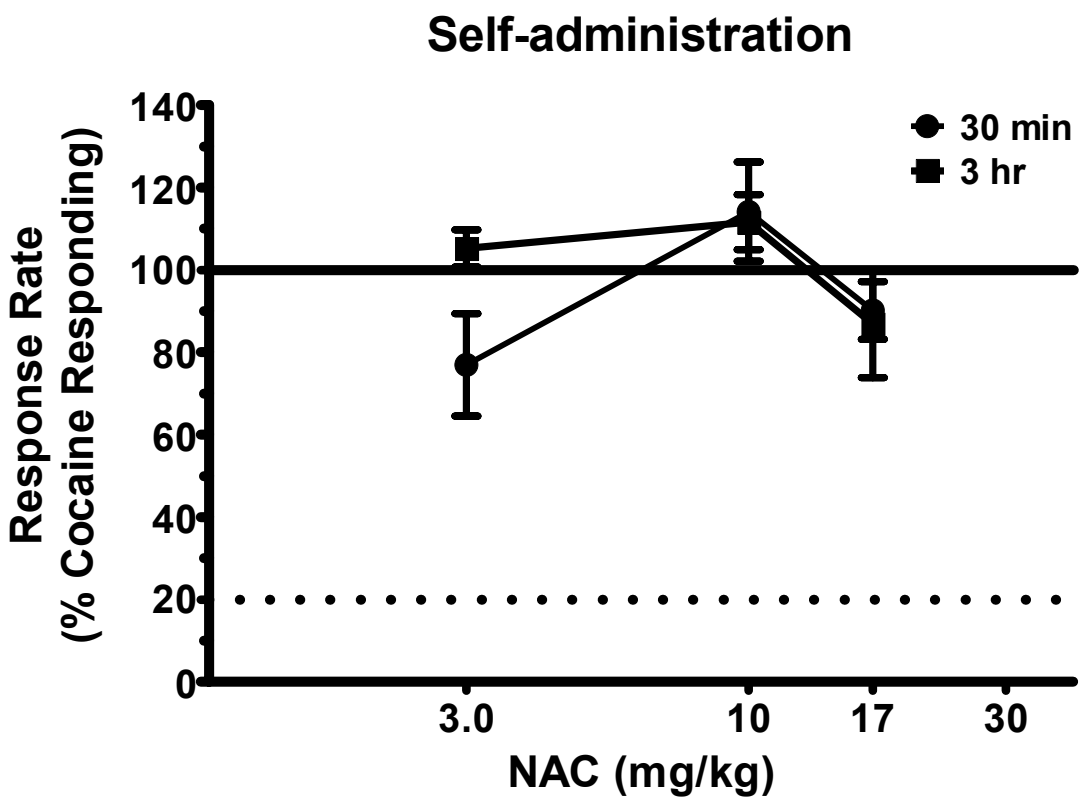
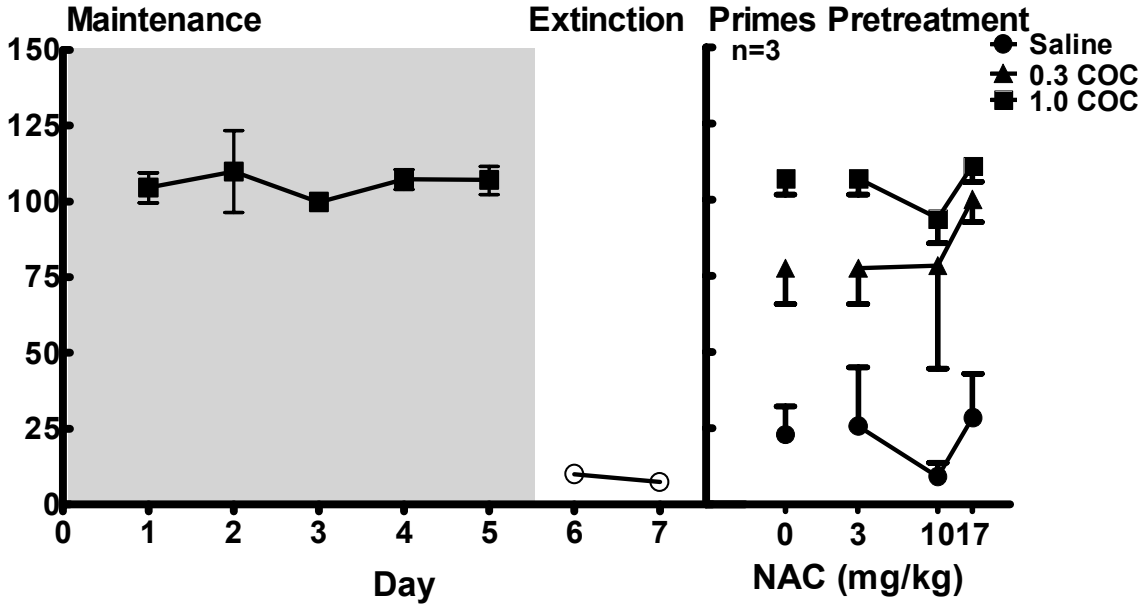

Self-administration
Response Rate
(% Cocaine Responding)
140
120
100
80
60
40
20
0
30 min
3 hr
3.0
10
17
30
NAC (mg/kg)

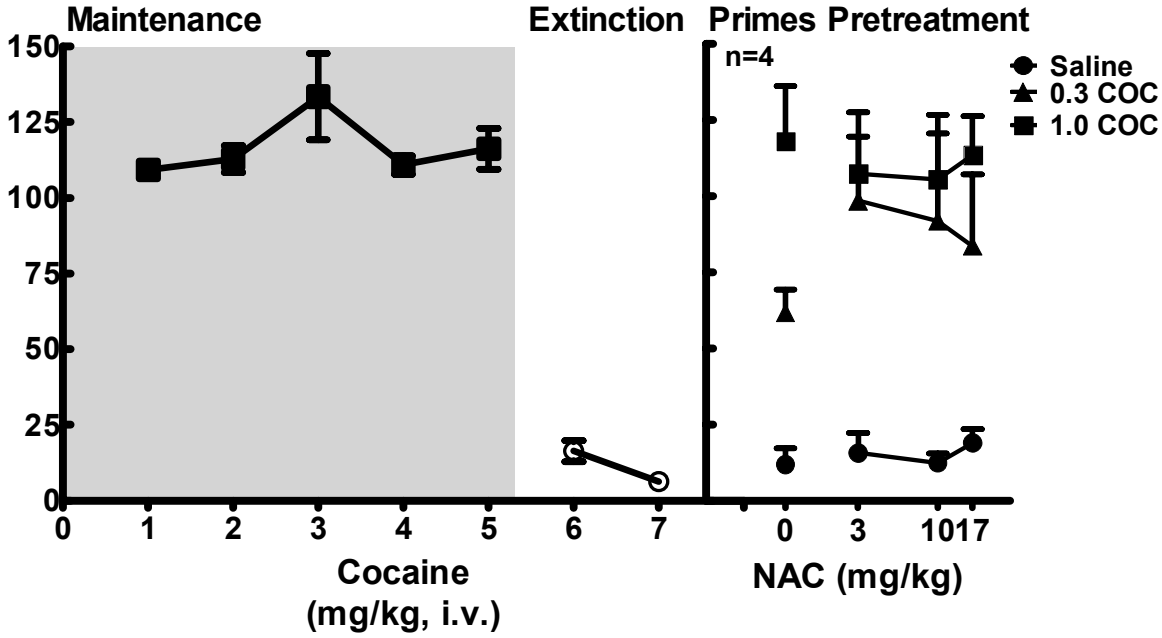
Figure 9 – Effects of NAC on reinstatement of previously extinguished cocaine self-administration

Cocaine self-administration prior to reinstatement sessions was stable, and extinguished readily within 2 sessions when saline was substituted for cocaine. Non-contingent injections of cocaine dose-dependently reinstated responding. However, NAC pretreatments did not significantly alter reinstatement of previously extinguished cocaine self-administration. Abscissa: day of treatment and pretreatment dose of LY379268 prior to reinstatement tests. Ordinate: rates of responding expressed as a percent of baseline control rates.

30 min NAC Reinstatment



3 hr NAC Reinstatment



V. EFFECTS OF mGluR2/3 MODULATION ON THE NEUROCHEMICAL AND BEHAVIORAL EFFECTS OF COCAINE

A. Introduction

Glutamate is the major excitatory neurotransmitter and is essential for many functions in the mammalian central nervous system (Watkins, 2000). Glutamate receptors can be divided into two classes: ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs) (Conn and Pin, 1997; Nakanishi, 1994a, b, 1992; Nakanishi and Masu, 1994; Nakanishi et al., 1994; Nakanishi et al., 1998; Nakanishi et al., 1996). Ionotropic glutamate receptors are not considered ideal targets for therapeutics because of their ubiquitous involvement in mediating fast synaptic transmission throughout the central nervous system. However, the G-protein coupled mGluRs have the capacity to modulate the actions of the ionotropic glutamate receptors as well as other neurotransmitter receptors, which make them good candidates for pharmacotherapeutic targets (Pin and Bockaert, 1995; Pin and Duvoisin, 1995). The mGluRs are part of the G-protein coupled receptor (GPCR) family 3, which includes the $GABA_B$ receptor, and are characterized by an extracellular N-terminal domain, a typical GPCR heptahelical domain, and an intracellular C-terminal tail of variable length (Bockaert and Pin, 1999; Schoepp, 2001). Eight mGluR subtypes have been identified and classified into three distinct groups based on pharmacology and sequence homology. Group I mGluRs (mGluR1 and 5) are positively coupled to phospholipase C. Group II mGluRs (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) are negatively coupled to adenyl cyclase. Group II mGluRs are primarily localized perisynaptically on presynaptic neurons and function as autoreceptors to regulate neurotransmitter release. Group II mGluRs have been associated with several neurological and psychiatric disorders including anxiety (Grillon et al., 2003; Muly et al., 2007; Yoshimizu et al., 2006),

schizophrenia and PCP-induced psychosis (Gupta et al., 2005; Imre et al., 2006; Lebois, 2008; Moghaddam, 2004; Woolley et al., 2008), and psychostimulant abuse (Pendyam et al., 2008; Peters and Kalivas, 2006; Uejima et al., 2007; Xi et al., 2002b).

Chronic administration of cocaine can alter glutamate transmission. In rats chronically treated with cocaine, there is a reported decrease in extracellular glutamate concentrations in the nucleus accumbens (Baker et al., 2003a; Bell et al., 2000; Hotsenpiller et al., 2001; Pierce et al., 1996). The reduction in basal levels of glutamate is associated with enhanced release of glutamate by an acute infusion of cocaine and may play an important role in cocaine-induced reinstatement of previously extinguished self-administration behavior (Baker et al., 2003a). A possible mechanism for the observed augmentation of glutamate release may be the decreased capacity of presynaptic mGluR2/3 to inhibit glutamate release. Therefore, it may be therapeutically beneficial to reduce glutamatergic signaling by targeting these receptors.

In rats, mGluR2/3 receptors are highly expressed in many regions of the brain including the prefrontal cortex, VTA, nucleus accumbens and striatum, areas which have been shown to play a pivotal role in addiction (Ohishi et al., 1994; Ohishi et al., 1993a, b; Petralia et al., 1996; Testa et al., 1998; Xi et al., 2002a). Studies have demonstrated a significant glutamatergic tone on mGluR2/3 receptors to suppress dopaminergic release in the nucleus accumbens (Hu et al., 1999). Furthermore, activation of mGluR2/3 receptors has been demonstrated to both decrease basal dopamine and the magnitude of dopamine response to systemic phencyclidine administration (Greenslade and Mitchell, 2004). Activation of mGluR2/3 receptors also attenuated behavioral responses believed to be mediated via dopaminergic pathways. Amphetamine-induced increases in locomotor activity were attenuated by pharmacological activation of mGluR2/3 with agonists treatment (David and Abraini, 2003), while locomotor activity was enhanced by selective mGluR2/3 antagonists (O'Neill et al., 2003). Furthermore, stimulation of

mGluR2/3 attenuated amphetamine self-administration in rats (Kim et al., 2005) and cocaine self-administration in nonhuman primates (Adewale et al., 2006). Activation of mGluR2/3 blocked cue-induced reinstatement of extinguished heroine self-administration in rats (Bossert et al., 2005) and drug-primed reinstatement of extinguished cocaine self-administration in rats (Peters and Kalivas, 2006) and nonhuman primates (Adewale et al., 2006). The current studies determined the effects of an mGluR2/3 agonist on cocaine-induced increases in extracellular dopamine in conscious nonhuman primates, as well as on the behavioral-stimulant and reinforcing effects of cocaine. It was hypothesized that pharmacological activation of mGluR2/3 would result in an attenuation of cocaine-induced increases in dopamine and this attenuation would be associated with decreased behavioral-stimulant and reinforcing properties of cocaine.

B. Results.

1. Microdialysis.

As previous studies have shown in squirrel monkeys, systemic administration of cocaine (1.0 mg/kg) produced a significant increase in extracellular dopamine as measured by in vivo microdialysis ($F[1,14]=14.284$, $p=0.032$, Figure 10). In drug interaction studies, subjects were given a 30-min pretreatment of the mGluR2/3 agonist, LY379268 (1.0 or 3.0 mg/kg). Administration of the high dose of LY379268 as a pretreatment before a saline injection did not significantly alter extracellular dopamine ($F[6,18]=2.316$, $p=0.078$). However, a one-way ANOVA demonstrated a significant main effect of pretreatment on the peak cocaine-induced increases in extracellular dopamine ($F[2,8]=7.767$, $p=0.013$). Post hoc analysis demonstrated that both doses tested of LY379268 significantly attenuated the peak increase in dopamine induced by cocaine. However, LY379268 did not completely abolish cocaine-induced increases in

extracellular dopamine. Additionally, the attenuation by LY379268 was not dose dependent, as the peak increases in extracellular dopamine were not significantly different when comparing between the two treatments. At the highest dose (3.0mg/kg), adverse side-effects were evident including weight loss, emesis and scratching behavior. Accordingly, higher doses were not examined.

2. Fixed-interval stimulus termination.

A cumulative-dosing paradigm was utilized to determine the effects of LY379268 on the behavioral-stimulant effects of cocaine. The response rates for individual subjects ranged from 0.19 to 0.47 responses/s with a mean (±S.E.M.) of 0.34 ± 0.13 responses/s. Cumulative dosing of saline produced no significant change in rates of responding throughout the session (Figure 11). However, increasing doses of cocaine produced a typical inverted U-shaped dose response curve with a significant main effect of dose ($F[4,30]=7.217$, $p=<0.001$). A 5-min pretreatment of LY379268 (1.0-3.0 mg/kg) had no significant effect when administered prior to repeated injections of saline ($F[12,60]=p=0.566$; Figure 11A), indicating that LY379268 alone had no behavioral effects. However, a 5-min pretreatment of LY372968 produced a significant downward shift in the cocaine dose response curve ($F[3,60]=1.204$, $p=0.002$; Figure 11B). A 30-min pretreatment of LY379268 did not significantly alter rates of responding during cumulative saline dosing ($F[12,60]= p=0.499$; Figure 11C). Interestingly, the downward shift of the cocaine dose response curve was no longer apparent when LY379268 was given with the longer pretreatment time ($F[3,60]=2.013$, $p=0.122$, Figure 11D).

To examine further the role of mGluR2/3 receptors in the behavioral-stimulant effects of cocaine, the selective mGluR2/3 antagonist, LY341495, was administered as a 5-min pretreatment. It was hypothesized that the antagonist would enhance the behavioral-stimulant effects of cocaine. However, pretreatment with LY341495 did not

significantly alter rates of responding following cumulative saline ($F[12,60]=0.543$, $p=0.877$) or cumulative cocaine ($F[12,60]=0.188$, $p=0.999$; Figure 12).

3. Self-administration.

Once stable cocaine self-administration was established under the second-order schedule, cocaine dose-response curves were determined for individual subjects. Subsequently, each subject was maintained on the unit dose that maintained peak rates of responding. The response rates for individual subjects ranged from 1.71 to 3.87 responses/s with a mean (±S.E.M.) of 2.34 ± 0.87 responses/s. Pretreatment with LY379268 for three consecutive days at each dose produced a significant decrease in rates of responding only at the 1.7 mg/kg dose [Mann-Whitney Rank Sum Test ($p=0.029$)] (Figure 13), though there was a trend towards an attenuation with the higher dose. It should be noted that frequent emesis was observed when the subjects were given the 3.0 mg/kg dose.

4. Reinstatement.

Subjects self-administered the dose of cocaine that maintained peak rates of responding for at least 5 consecutive test sessions with no more than 20% variability between sessions (Figure 14). The response rates for individual subjects ranged from 1.75 to 3.88 responses/s with a mean (±S.E.M.) of 2.37 ± 0.22 responses/s. Cocaine was then substituted for saline until rates of responding were extinguished to less than 30% of the rates maintained by cocaine ($F[1,3]= 6583.213$, $p=<0.001$). All subjects extinguished within two days of saline substitution. Initially, subjects were administered a 5-min vehicle pretreatment prior to the reinstatement prime (vehicle or cocaine, 0.3, or 1.0 mg/kg) administration, which was administered 5-min prior to the start of the session. Once baseline reinstatement behavior was established, subjects were

administered LY379268 (1.0 or 1.7 mg/kg) 5-min prior to the reinstatement prime. Cocaine significantly increased rates of responding at the doses tested ($F[2,6]= 23.980$, $p=0.001$, Figure 14). Neither dose of LY379268 administered significantly altered cocaine-induced reinstatement, although the effect of LY379268 in combination with 1.0 mg/kg cocaine approached significance ($F[2,5]=5.272$, $p=0.059$). Because of the emesis observed in other experiments in this study with the 3.0 mg/kg LY379268, this dose was not administered as a pretreatment to reinstatement.

C. Discussion

Dysregulation of the basal glutamatergic tone has been attributed as one of the underlying mechanisms for cocaine relapse and addiction. Baker and Kalivas reported that the origin of extracellular dopamine was primarily from the cystine-glutamate transporter localized on astrocytes (Baker et al., 2002b). Acute administration of cocaine increased extracellular glutamate, potentially due to increased vesicular glutamate release. However, chronic cocaine decreased basal glutamate and this long-lasting dysregulation has been proposed to be a potential mechanism that underlies the enduring propensity for relapse of cocaine addicts even after long bouts of abstinence (Baker et al., 2002a). Basal glutamate provides glutamatergic tone on extrasynaptic glutamate receptors, such as the mGluR2/3, that regulate synaptic release of glutamate (Xi et al., 2002a). In rodents undergoing chronic cocaine administration and withdrawal, cocaine induces an enhanced increase in extracellular glutamate, suggesting that the basal glutamatergic tone on mGluR2/3 may be an important modulator in the behavioral pharmacology of cocaine.

The purpose of the current studies was to determine the effect of manipulating mGluR2/3 on cocaine-induced changes in dopamine neurochemistry in conscious nonhuman primates. Furthermore, the current studies aimed to determine if changes in

dopamine neurochemistry would be reflected in changes in the behavioral-stimulant and reinforcing effects of cocaine. Administration of the mGluR2/3 agonist, LY379268, attenuated cocaine-induced increases in extracellular dopamine without directly altering basal extracellular dopamine. There are conflicting reports regarding the effects of activation of mGluR2/3 receptors on basal extracellular dopamine in rodents. Early studies demonstrated increases in basal levels of dopamine by selective mGluR2/3 agonists in the prefrontal cortex (Pintor et al., 1998), nucleus accumbens (Ohno and Watanabe, 1995; Taber and Fibiger, 1995), and striatum (Cartmell et al., 2000c). Conversely, Greenslade and Mitchell (2004) demonstrated that LY379268 could block phencyclidine induced increases in dopamine in the rat nucleus accumbens, but only at doses in which LY379268 also decreased basal dopamine. Further, activation of group II mGluR receptors produced a biphasic effect on dopamine, with low and high doses having little effect and the intermediate dose decreasing extracellular dopamine in the nucleus accumbens (Moghaddam and Adams, 1998). The effects of LY379268 on basal extracellular dopamine have not been reported previously in nonhuman primates.

Administration of LY379268 significantly attenuated the behavioral-stimulant effects of cumulative doses of cocaine when administered as a 5-min pretreatment. Because the largest attenuation was seen at the peak dose of cocaine (0.3 mg/kg) and this dose was administered approximately 30-min into the session, a longer pretreatment time was utilized to evaluate the potential influence of drug time course of LY372968. When LY379268 was administered as a 30-min pretreatment, an attenuation of the behavioral-stimulant effects of cocaine was no longer evident. This suggests that the effectiveness of the agonist to attenuate the behavioral-stimulant effects of cocaine dissipated over the course of the test session following a longer pretreatment time. Conversely, blocking the mGluR2/3 receptors with the antagonist, LY341495, had no effect on the behavioral-stimulant effects of cocaine. This is

consistent with previous studies in rodents which demonstrated that LY379268 attenuated amphetamine-induced ambulations and rearing in rats, while LY341495 did not significantly alter ambulations and rearing in rats following administration of vehicle or d-amphetamine (Cartmell et al., 2000a). This suggests that endogenous glutamatergic tone on mGluR2/3 receptors may not be critical to the behavioral-stimulant effects of cocaine.

In contrast to previous studies in rodents and nonhuman primates demonstrating a significant and dose-dependent attenuation of cocaine self-administration, the effects of LY379268 on the reinforcing properties of cocaine were not robust or consistent across doses. LY379268 significantly attenuated cocaine self-administration, but only at the 1.7 mg/kg dose, though there was trend toward a decrease in cocaine self-administration with the 3.0 mg/kg dose. The observed effects of LY379268 were not likely due to a nonspecific disruption of behavior given that LY379268 administered alone had no significant effect on behavior maintained by the fixed-interval schedule of stimulus termination. Importantly, administration of LY379268 resulted in a strong trend toward attenuating cocaine-induced reinstatement of previously extinguished self-administration that approached significance. Although less robust, these results are consistent with previous studies in which LY379268 dose-dependently attenuated cocaine-primed reinstatement in rats (Peters and Kalivas, 2006) and in nonhuman primates (Adewale et al., 2006). It is unknown whether gender, genetic variables or drug history may influence the effectiveness of LY379268 in altering the reinforcing properties and reinstatement effects of cocaine.

Recent studies have demonstrated that LY379268 may also have partial agonist activity at dopamine D2 receptors (Seeman et al., 2008). Accordingly, it is unclear whether the current observations are due, in part, to the effects of LY379268 at

dopamine D2 receptors. Further studies utilizing selective antagonists for dopamine receptors and mGluR2/3 will elucidate which receptors mediate the effects of LY379268 on the pharmacology of cocaine. Compounds with higher potency and increased selectivity for the mGluR2/3 have been developed (Rorick-Kehn et al., 2005) and may be useful in isolating the effects of mGluR2/3 on cocaine pharmacology. Additionally, LY379268 does not distinguish between mGluR2 and mGluR3. Increasing evidence suggests that selective mGluR2 (Corti et al., 2007; Fell et al., 2008; Rowe et al., 2008) activation may be a more effective target for pharmacotherapeutics of psychiatric disorders such as anxiety and schizophrenia. Dopamine and glutamate interactions appear to play a significant in the underlying mechanisms of anxiety and schizophrenia, thus, these selective potentiators of mGluR2 may also be more effective in attenuating the behavioral-stimulant and reinforcing effects of cocaine.

The current studies provide the first evidence that LY379268 attenuates the neurochemical effects of cocaine in nonhuman primates. Though the effects of LY379268 administration in the behavioral pharmacology of cocaine were not as robust as in previously reported studies, these data further support the need to develop better pharmacological tools to manipulate mGluR2/3 as potential targets for pharmacotherapeutics of cocaine addiction.

D. Figures for the effects of LY379268 on the neurochemical and behavioral effects of cocaine

Figure 10 – Effects of LY379268 on dopamine neurochemistry

Systemic administration of cocaine (1.0mg/kg) significantly elevated extracellular dopamine levels in the caudate nucleus in four squirrel monkeys. The peak response was achieved within 20-min and returned to baseline within 60-min. In contrast, LY379268 (1.0-3.0 mg/kg) administration did not significantly alter extracellular dopamine when administered as a pretreatment to saline. However, LY379268 pretreatments significantly attenuated cocaine-induced increases in extracellular dopamine. The 10-min sampling intervals indicated began 60-min post probe insertion. Arrows indicate the points at which the pretreatment, cocaine or saline were administered. Data points represent mean±SEM dopamine levels as a percent of values obtained prior to drug administration. Abscissa: time. Ordinate: extracellular concentrations of dopamine expressed as a percent of baseline control levels. (* denotes significant change in extracellular dopamine compared to vehicle administration. ** denotes significant change in extracellular dopamine compared to vehicle administration and to cocaine administered alone.)

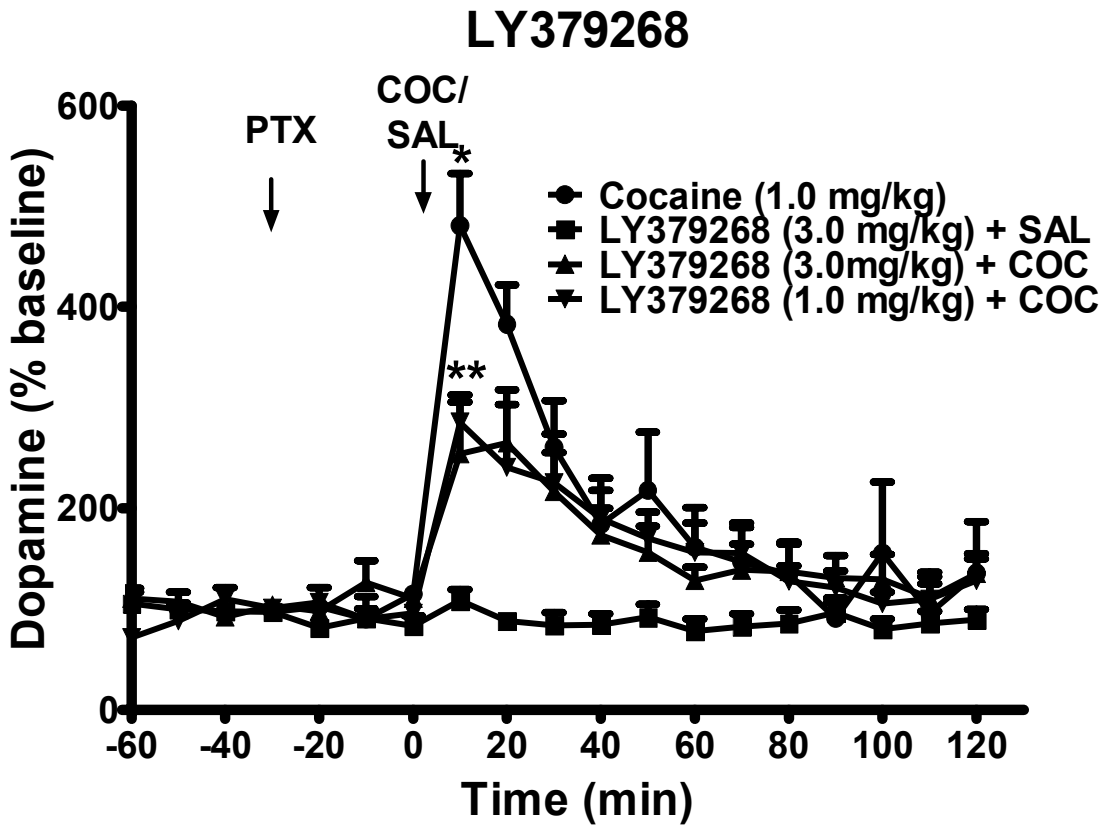

LY379268
PTX
COC/
SAL
*
**
Cocaine (1.0 mg/kg)
LY379268 (3.0 mg/kg) + SAL
LY379268 (3.0mg/kg) + COC
LY379268 (1.0 mg/kg) + COC
Dopamine (% baseline)
600
400
200
0
-60
-40
-20
0
20
40
60
80
100
120
Time (min)

Figure 11 – Effects of LY379268 on the behavioral-stimulant effects of cocaine Cumulative infusions of saline did not significantly alter baseline rates of responding maintained by a fixed-interval schedule of stimulus termination (A). Cumulative doses of cocaine (0.03-1.0 mg/kg) produced a typical inverted-U shaped dose effect curve (B). Both 0.1 and 0.3 mg/kg cocaine produced significant increases in rates of responding. LY379268 (1.0-3.0 mg/kg) pretreatments did not significantly alter rates of responding maintained by a fixed-interval avoidance schedule when cumulative doses of saline were administered (i.v.) at set intervals throughout the session (A,C). However, a 5-min LY379268 pretreatment produced a downward shift of the cocaine dose effect curve (B). In contrast, a 30-min pretreatment time was ineffective in attenuating the behavioral-stimulant effects of cocaine (D). Abscissae: drug dose or saline infusion. Ordinates: rates of responding expressed as a percent of baseline control rates. (* denotes significant change in rates of responding when comparing cocaine administration to vehicle administration. ** denotes significant change in rates of responding during the cocaine cumulative dose response curve when comparing LY379268 pretreatment to a vehicle pretreatment.)

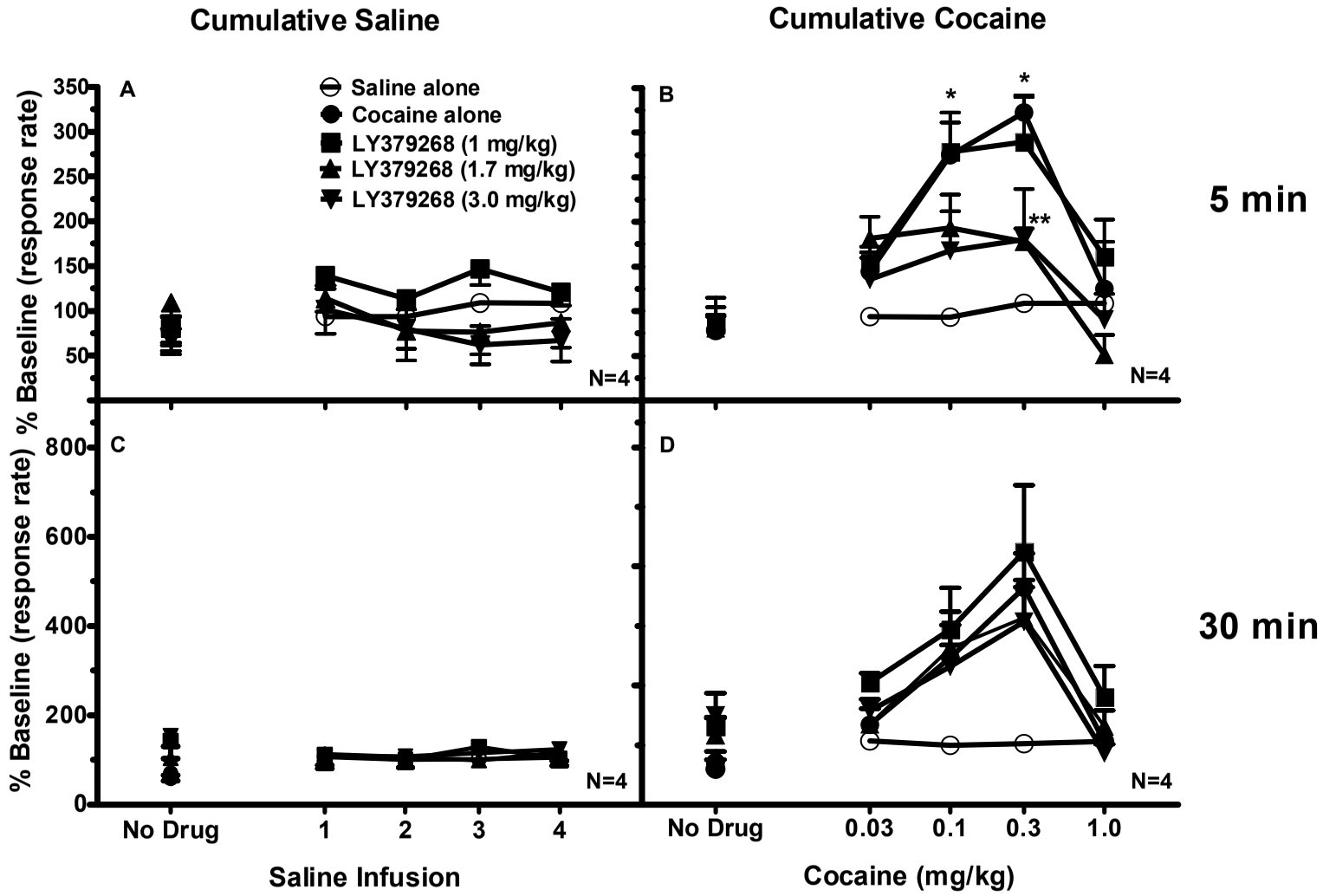

Cumulative Saline
Cumulative Cocaine
A
B
C
D
Saline alone
Cocaine alone
LY379268 (1 mg/kg)
LY379268 (1.7 mg/kg)
LY379268 (3.0 mg/kg)
% Baseline (response rate)
350
300
250
200
150
100
50
800
600
400
200
0
N=4
*
**
5 min
30 min
No Drug
1
2
3
4
0.03
0.1
0.3
1.0
Saline Infusion
Cocaine (mg/kg)

Figure 12 – Effects of LY341495 on the behavioral-stimulant effects of cocaine LY341495 (1.0-10.0 mg/kg) pretreatments did not significantly alter rates of responding maintained by a fixed-interval schedule of stimulus termination when saline was administered at set intervals throughout the session (A). Moreover, LY341495 pretreatments did not significantly alter the behavioral-stimulant effects of cocaine (B). Abscissae: drug dose or saline infusion. Ordinates: rates of responding expressed as a percent of baseline control rates.

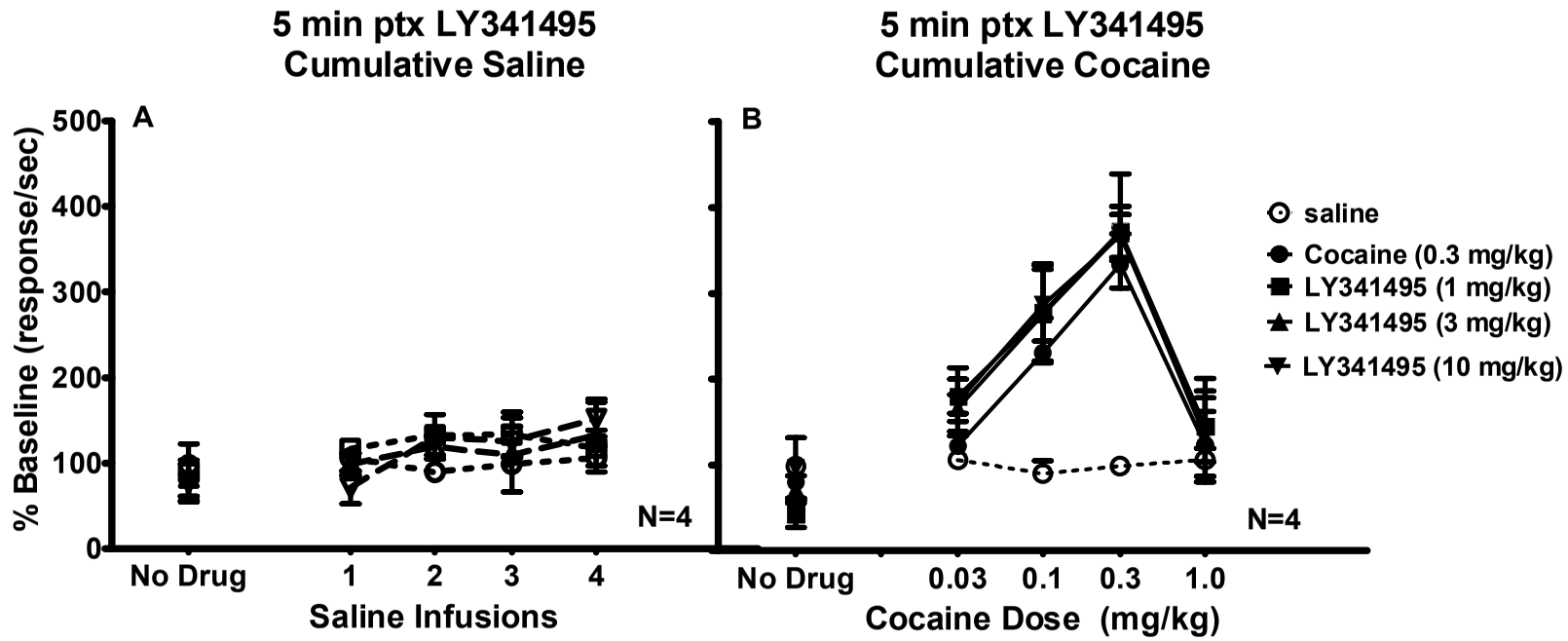

5 min ptx LY341495
Cumulative Saline
5 min ptx LY341495
Cumulative Cocaine
A
B
% Baseline (response/sec)
500
400
300
200
100
0
N=4
N=4
No Drug
1
2
3
4
Saline Infusions
No Drug
0.03
0.1
0.3
1.0
Cocaine Dose (mg/kg)
saline
Cocaine (0.3 mg/kg)
LY341495 (1 mg/kg)
LY341495 (3 mg/kg)
LY341495 (10 mg/kg)

Figure 13 – Effects of LY379268 on cocaine self-administration

Each dose of LY379268 was administered for three consecutive days on separate occasions. Administration of LY379268 significantly decreased responding maintained by a second-order schedule of cocaine self-administration, but only at the intermediate dose. However, the high dose demonstrated a strong trend toward attenuating self-administration rates. Abscissa: drug dose. Ordinate: rates of responding expressed as a percent of baseline control rates. (* denotes a significant change in rates of responding compared to saline pretreatment. The bold line denotes the baseline rates of responding for the dose of cocaine that maintained peak rates of responding, while the dashed line denotes the extinction rates of responding when saline was substituted for cocaine.)

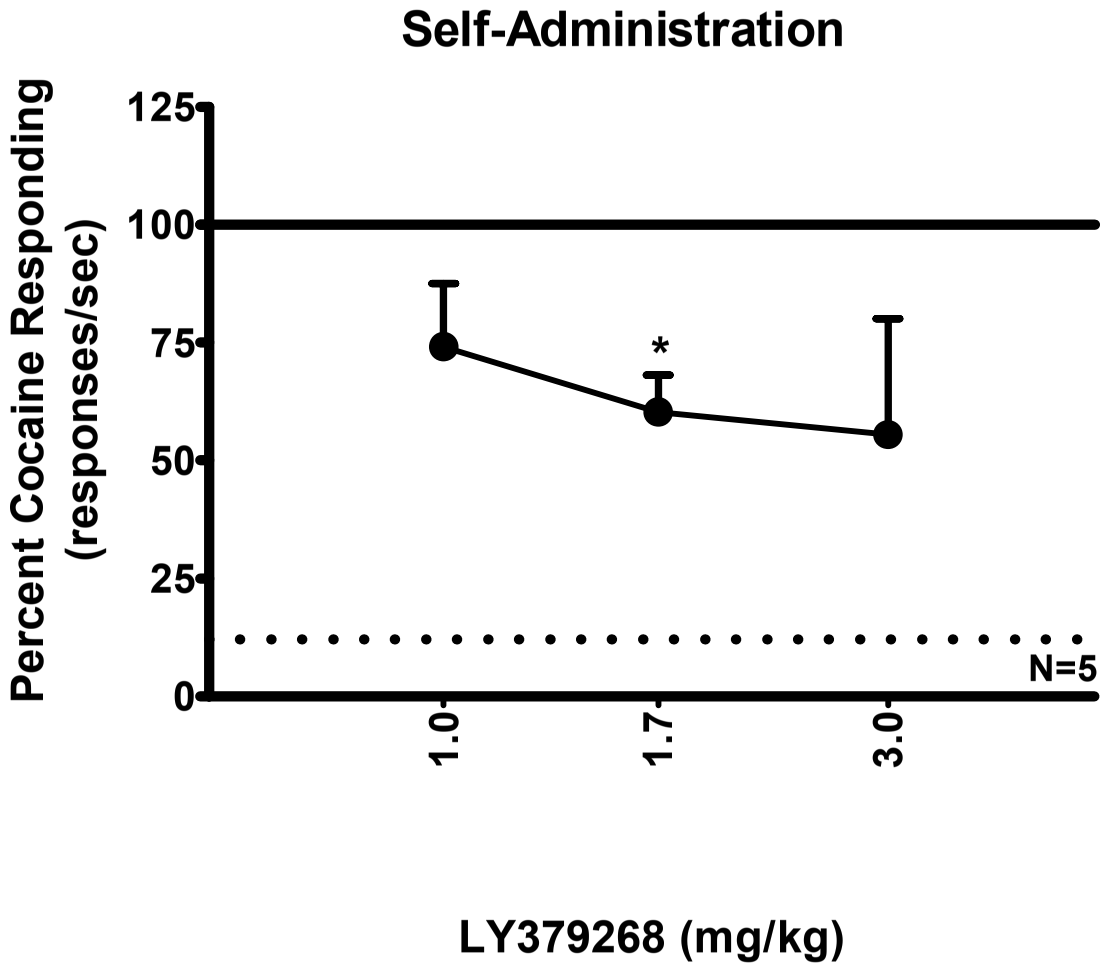
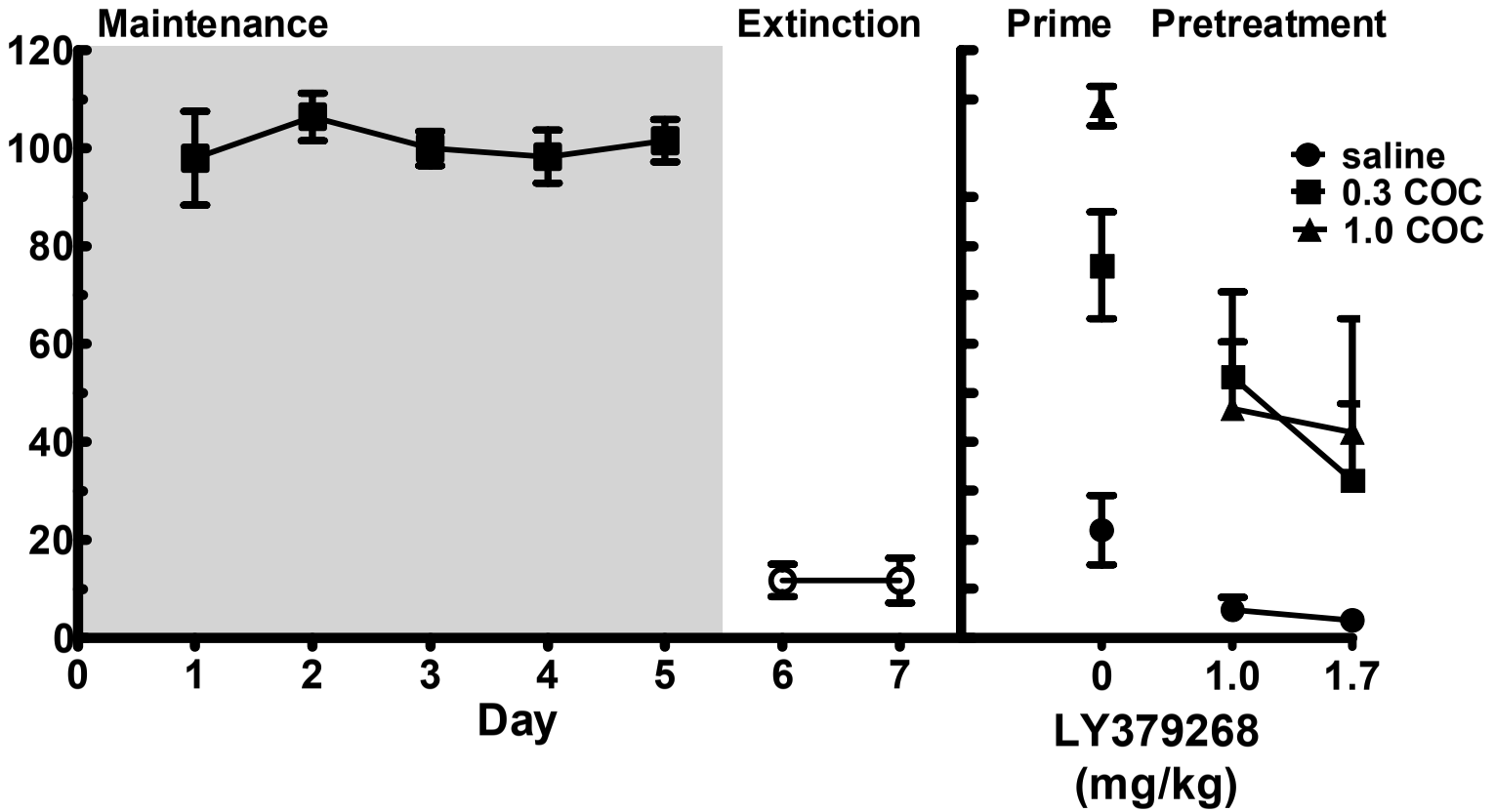

Self-Administration
Percent Cocaine Responding
(responses/sec)
125
100
75
50
25
0
*
N=5
1.0
1.7
3.0
LY379268 (mg/kg)

Figure 14 – Effects of LY379268 on cocaine-induced reinstatement.
Cocaine self-administration was reliable during the maintenance phase, and extinguished readily in 2 sessions. Subsequently, non-contingent injections of cocaine (0.3 and 1.0 mg/kg) reinstated responding. LY379268 pretreatment demonstrated a strong trend toward significantly attenuating reinstatement of responding induced by the high dose (1.0 mg/kg) of cocaine. Abscissa: day of treatment and pretreatment dose of LY379268 prior to reinstatement tests. Ordinate: rates of responding expressed as a percent of baseline control rates.


Maintenance
Extinction
Prime
Pretreatment
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day
0
1.0
1.7
LY379268
(mg/kg)
saline
0.3 COC
1.0 COC

VI. Discussion

The goal of the current studies was to characterize the role of mGluR2/3 receptors on dopamine neurochemistry and the behavioral pharmacology of cocaine in squirrel monkeys. To achieve this goal, mGluR2/3 receptors were targeted in two ways, indirectly by increasing basal extracellular glutamate levels via the cystine-glutamate transporter and directly with a selective mGluR2/3 agonist. Previous studies in rodents demonstrated a direct correlation between enhancing cystine-glutamate transporter and mGluR2/3 receptor activation in the behavioral pharmacology of cocaine (Baker et al., 2002a; Manzoni et al., 1997; Xi et al., 2002a). Basal glutamate is regulated by the cystine-glutamate transporter (Baker et al., 2002b), thus the cystine-glutamate transporter has been investigated as a potential target for cocaine pharmacotherapeutics in both rodent (Baker et al., 2003a; Baker et al., 2003b; Baker et al., 2002a; Kau et al., 2008; Madayag et al., 2007; Moran et al., 2005; Moussawi et al., 2009) and human studies (LaRowe et al., 2006; Mardikian et al., 2007). In the current studies, the objective was to extend the findings in rodent studies to nonhuman primates, as the nonhuman primate brain is more complex and would determine the generality of the effects observed in rodents across mammalian species. Furthermore, studies have shown that the cystine-glutamate transporter regulates glutamate synaptic activity via the mGluR2/3 receptors (Moran et al., 2005). Enhancing cystine-glutamate transporter activity in the nucleus accumbens of rats decreased extracellular glutamate and this effect was blocked by a selective mGluR2/3 receptor antagonist. Thus, the mGluR2/3 receptors may be a more selective target for cocaine pharmacotherapeutics. Accordingly, the current studies determined if the relationship between the cystine-glutamate transporter and the mGluR2/3 receptors would generalize to nonhuman primates. Initially, the activation of cystine-glutamate transporters with the cystine prodrug, NAC,

on dopamine neurochemistry was measured using microdialysis and the effects of NAC on the behavioral-stimulant and reinforcing effects of cocaine were measured using a fixed-interval schedule of stimulus termination and second-order schedule of self-administration, respectively. Subsequently, the same measures were utilized to measure the effects of mGluR2/3 receptor activation on dopamine neurochemistry and the behavioral pharmacology of cocaine. It was hypothesized that the effects of enhancing the cystine-glutamate transporter on cocaine pharmacology was mediated through activation of the mGluR2/3 receptors. Thus, directly activating mGluR2/3 receptors should have the same effects on the pharmacology of cocaine as enhancing cystine-glutamate transporter activity.

The current studies provide the first evidence that administration of the cystine prodrug, NAC, or the mGluR2/3 agonist, LY379268, significantly attenuated cocaine-induced increases in extracellular dopamine without directly altering basal extracellular dopamine. Neither NAC or LY379268 alone significantly alter basal dopamine. Though both drugs attenuated the effects of cocaine on extracellular dopamine, neither treatment completely abolished the effects of cocaine. With either pretreatment, cocaine still induced approximately a 200-250% increase in extracellular dopamine compared to baseline. This increase in dopamine is equivalent to a 0.3 mg/kg dose of cocaine, which is a dose that is highly reinforcing and induces behavioral-stimulant effects in nonhuman primates. Thus, it is not surprising that the attenuation of cocaine-induced increases in extracellular dopamine by NAC or LY379268 were either not reflected or marginally reflected, respectively, in changes in the behavioral pharmacology of cocaine by pretreatments. A more significant attenuation of extracellular dopamine may be necessary for changes in behavior to be observed.

The hypothesis at the beginning of these studies was that a pretreatment of NAC or LY379268 would attenuate the behavioral effects of cocaine. The observed

attenuation of cocaine-induced increases in extracellular dopamine provided further evidence that a pretreatment of NAC or LY379268 would attenuate the behavioral effects of cocaine. In contrast to the rodent studies, NAC had no significant effect on the behavioral pharmacology of cocaine in the operant tasks utilized despite the variation of dose, route of administration, and pretreatment time. The effects of LY379268, though not as robust, were consistent with previous observations. An attenuation of the behavioral-stimulant effects of cocaine was observed with a short pretreatment of LY379268 that was no longer evident with a longer pretreatment of LY379268. This suggests that the effectiveness of the agonist, LY379268, on attenuating the behavioral-stimulant effects of cocaine dissipated over the course of the test session following a longer pretreatment time. Conversely, blocking the mGluR2/3 receptors with the antagonist, LY341495, had no effect on the behavioral-stimulant effects of cocaine. This is consistent with previous studies that demonstrated that LY379268 attenuated amphetamine-induced ambulation and rearing in rats, while LY341495 did not significantly alter d-amphetamine-induced ambulation and rearing in rats (Cartmell et al., 2000a). This suggests that the endogenous glutamatergic tone on mGluR2/3 receptors may not be critical to the interactions observed with cocaine. The effects of LY379268 on the reinforcing properties of cocaine, though not robust, were also consistent with previous reports. LY379268 significantly attenuated cocaine self-administration, but only at the 1.7 mg/kg dose, though there was trend toward a decrease with a higher pretreatment dose. The observed attenuation by LY379268 pretreatment was not likely due to a nonspecific disruption of behavior given that LY379268 administered alone had no significant effect on behavior maintained by the fixed-interval schedule of stimulus termination. Importantly, administration of LY379268 resulted in a strong trend toward attenuating cocaine-induced reinstatement of previously extinguished self-administration that approached significance. Thus, the current results

were consistent with previous studies that demonstrate that LY379268 dose-dependently attenuated cocaine-primed reinstatement in rats (Peters and Kalivas, 2006) and in nonhuman primates (Adewale et al., 2006). Collectively, these results suggest that the cystine-glutamate transporter would not be as effective as a pharmacotherapeutic target for cocaine addiction as the mGluR2/3 receptors.

The lack of effectiveness of NAC on the behavioral effects of cocaine raises important issues. The current experimental design attempted to extend previous finding in rodents to a nonhuman primate model. The current studies suggest a species difference. However, other factors may also explain the discrepancy observed between these results and results from previous studies. The current studies were conducted under the assumption that NAC was enhancing extracellular glutamate via the cystine-glutamate transporter. Without neurochemical evidence of NAC enhancing extracellular glutamate in the nonhuman primate, it is difficult to establish if NAC was, indeed, influencing the cystine-glutamate transporter. Systemic administration of the drugs utilized during this study did not induce changes in extracellular glutamate as measured by HPLC with tandem mass spectrometry. Unfortunately, the results were equivocal and further raised questions because the methods used in the current study were different from those used in previous studies. Hence, there exists the possibility that the current methodology utilized was not sensitive enough to measure changes in glutamate. A two-pronged approach was utilized to address these possibilities. First, NAC was administered directly into the caudate nucleus and the nucleus accumbens via reverse dialysis in squirrel monkeys. The caudate is a large area of the brain in comparison to the microdialysis probe and extracellular glutamate is tightly regulated. Thus, changes in extracellular glutamate may be difficult to observe with systemic administration of NAC. Direct administration allowed NAC to interact with the cystine-glutamate transporter at the site where glutamate was being measured. Second, previous studies in

rodents were replicated but glutamate was measured in the striatum instead of the nucleus accumbens using HPLC with mass spectrometry. Previous studies in rodents focused exclusively on the effects of NAC in the nucleus accumbens; however the current studies were conducted in the caudate nucleus. Conducting these experiments in the same brain region in rodents and nonhuman primates distinguished species differences from regional differences. In addition, it validated the use of HPLC with tandem mass spectrometry as a method to measure glutamate.

Direct administration of NAC into either the caudate or nucleus accumbens did not significantly increase extracellular glutamate in nonhuman primates. Though not significant, there was a trend toward an increase with the lowest dose of NAC in both brain regions. Interestingly, there appeared to be a difference in glutamate regulation between the caudate and the nucleus accumbens. There appeared to be tighter regulation of glutamate in the caudate than in the nucleus accumbens, as there was a tendency to see a slight increase in extracellular glutamate with each subsequent dose of NAC in the accumbens, while in the caudate there was only a trend toward an increase with the lowest dose. This observation is interesting because studies have shown that in nonhuman primates the dorsal and ventral striatum pathways are not as distinct as in the rodents (Haber et al., 2000; Haber and McFarland, 1999; Lynd-Balta and Haber, 1994a, c). These pathways may be much more disperse in the the nonhuman primate brain than in the rodents, but feedback and regulatory mechanisms may be distinctly different in different regions of the brain. These pathways are much more complex and interconnected in the nonhuman primate brain, which emphasizes the importance of testing the generality of observations derived from rodent models. Though the pathways themselves are interconnected, understanding differential regulatory processes would allow for more specific targeting of specific brain regions for pharmacotherapeutics.

Further studies on the regulation of extracellular glutamate in different brain regions may be important for understanding the interactions between glutamate and dopamine.

As observed with local administration of NAC, increasing doses of the glutamate reuptake inhibitor, THA, did not induce significant changes in extracellular glutamate though there was a trend toward an increase with the lowest dose. When the highest dose was administered alone, there was a small but significant increase in extracellular glutamate. This suggests that the first dose induced compensatory mechanisms in regulating glutamate that prevented subsequent infusions of THA from inducing changes in extracellular glutamate. Because glutamate is so tightly regulated, a more rapid method of measuring glutamate such as real-time voltammetry or enzyme-immobilized biosensors would be more effective in visualizing the changes in glutamate induced by NAC.

Importantly, the microdialysis studies conducted in rodents receiving repeated administration of cocaine provided strong evidence that the lack of effectiveness of NAC on the behavioral pharmacology of cocaine in nonhuman primates was due to a species difference. These results were consistent with previous studies done in the nucleus accumbens, in which NAC only induced an increase in extracellular glutamate in subjects that were chronically treated with cocaine and withdrawn. These results suggest that the effects of NAC generalize across brain regions in rodents where the ventral and dorsal striatum play distinctly different roles in the mechanism underlying the behavioral effects of cocaine. Furthermore, these data were consistent with previous data measured with HPLC with florescence detection. This provides strong evidence that the lack of effects of systemic administration of the compounds of interest on extracellular glutamate were not due to differences in methodologies used. Collectively, these data suggest that the lack of effect of NAC in squirrel monkeys may be due to glutamate being

more tightly regulated in the nonhuman primate as compared to rodents. Higher order processing may require that glutamate be more highly regulated to prevent excitotoxicity.

A number of factors may contribute to the lack of effectiveness of NAC on the behavioral effects of cocaine. It is important to note that NAC indirectly activates mGluR2/3 by enhancing extracellular glutamate through increased cystine-glutamate transport. A global increase in extracellular glutamate should not only enhance mGluR2/3 activation, but enhance activation of all extrasynaptic glutamatergic receptors. Group I mGluR1/5 receptors are predominantly localized perisynaptically on postsynaptic neurons and are highly localized in the same areas as the mGluR2/3 receptors. Group I mGluR receptors are G_q-coupled and increase phospholipase C activation and have the capacity to positively modulate glutamate activation. The mGluR5 receptor has been demonstrated to play a role in the stimulant and reinforcing properties of cocaine (Backstrom and Hyytia, 2006; Chiamulera et al., 2001; Lee et al., 2004; McGeehan et al., 2004; McGeehan and Olive, 2003). mGluR5 knock-out mice do not exhibit the behavioral-stimulant and reinforcing properties of cocaine (Chiamulera et al., 2001) suggesting a significant role of mGluR5 in the behavioral effects of cocaine. Pharmacological blockade of mGluR5 also significantly attenuated the behavioral effects of cocaine in rodents and nonhuman primates (Backstrom and Hyytia, 2006; Lee et al., 2004; McGeehan et al., 2004; McGeehan and Olive, 2003). Thus, activation of mGluR5 through increased glutamatergic tone may mask the effects of mGluR2/3 activation. This would also explain why selective activation of mGluR2/3 receptors was more effective in altering the behavioral effects of cocaine. Both mGluR3 and mGluR5 are also localized on astrocytes, though mGluR3 is the predominant mGluR receptor localized on astrocytes (Aronica et al., 2000). Both of these receptors have been shown to upregulate in conditions of high excitation such as electrical stimulation (Aronica et al., 2000).

Thus, increased extracellular glutamate may also induce upregulation of these receptors and stimulate regulatory pathways in astrocytes to prevent excitotoxicity.

High extracellular concentrations of glutamate are neurotoxic, so it is necessary to strictly regulate glutamate in the brain. The X_{AG}^- superfamily of Na^+-dependent glutamate transporters rapidly take up glutamate. There are five subtypes of these excitatory amino acid transporters (EAAT) differentiated by structure and localization. These transporters maintain the intracellular glutamate concentration approximately 10,000 fold greater than in the extracellular compartment. Inside the astrocytes, L-cystine is immediately reduced in the reductive intracellular environment to L-cysteine, maintaining approximately 100-fold greater concentration of L-cystine in the extracellular compartment than the intracellular concentration (Murphy et al., 1989). Thus, glutamate and cystine move passively along a concentration gradient. It is assumed that NAC is increasing extracellular glutamate by being deacylated to the amino acid L-cysteine, which is readily oxidized in the extracellular environment to L-cystine and increasing passive transport. However, the transporter is bidirectional and increasing concentrations of extracellular glutamate can also induce uptake of glutamate via the transporter. Based on how concentrations of glutamate and cystine are maintained, this is unlikely, but cannot be dismissed as a contributing factor of the lack of effect of NAC. Moreover, the cystine-glutamate transporter and EAAT work cooperatively to protect neurons from glutamate excitotoxicity (Lewerenz et al., 2006). Thus, compensatory mechanisms to prevent glutamate excitotoxicity may mask the effects of NAC on the behavioral pharmacology of cocaine.

Cocaine itself has been demonstrated to induce oxidative stress (Dietrich et al., 2005; Pacifici et al., 2003) by inducing the generation of reactive oxygen species. Increased reactive oxygen species induces an increase in the generation of glutathione. Glutathione is a major cellular antioxidant and is important for protection against

oxidative stress. The cystine-glutamate transporter is important for maintaining glutathione levels, as cysteine is the rate-limiting substrate for making glutathione. Though in vivo excitotoxicity has not yet been demonstrated, NAC is excitotoxic in cultured cells. NAC is initially converted to cysteine and extracellular cysteine can induce oxidative stress and potentiate glutamate toxicity in rats (Puka-Sundvall et al., 1995). L-cysteine is unstable as a free amino acid and is readily broken down in the blood and reduced to cystine. However, L-cysteine can also be taken up by EAAT3, localized on neurons, such as dopamine neurons to produce glutathione and prevent oxidative damage (Chen and Swanson, 2003). This would decrease the amount of cysteine that is converted to cystine reducing the potential for NAC to enhance cystine-glutamate transport. Once cysteine is converted to cystine, EAAT3 does not take up cystine. Increased oxidative stress would probably enhance mechanisms necessary for antioxidant activity in neurons. A combination of NAC and cocaine may result in an extracellular environment that would induce increases in antioxidant activity in neurons.

The ineffectiveness of NAC on the behavioral effects of cocaine provides further evidence that the endogenous glutamatergic tone on mGluR2/3 receptors may not be critical to the cocaine-induced behaviors observed in the current studies in nonhuman primates. This is in stark contrast to the rodent model. Rodent studies have shown that NAC administration prevents reinstatement of extinguished cocaine self-administration (Baker et al., 2003a; Kau et al., 2008; Moran et al., 2005; Zhou and Kalivas, 2007) and that this attenuation can be blocked by selective mGluR2/3 antagonists (Moran et al., 2005). This discrepancy suggests that LY379268 may modulate the behavioral effects of cocaine via pathways other than by its effects on mGluR2/3 receptors. Recent studies have demonstrated that LY379268 may also have partial agonist activity at dopamine D2 receptors (Seeman et al., 2008). Thus, LY379268 may have been more effective than NAC, in part, because of the effects of LY379268 at dopamine D2 receptors. Further

studies, utilizing selective antagonists for dopamine receptors and mGluR2/3 would elucidate which receptors mediate the effects of LY379268 on the pharmacology of cocaine. Compounds with higher potency and increased selectivity for the mGluR2/3 have been developed (Rorick-Kehn et al., 2007; Rorick-Kehn et al., 2005) and may be useful in isolating the effects of mGluR2/3 on cocaine pharmacology. Additionally, LY379268 does not distinguish between mGluR2 and mGluR3. mGluR2 knock out mice demonstrate an increased responsiveness to cocaine administration (Morishima et al., 2005), suggesting that mGluR2 may play a significant role in the underlying mechanisms of cocaine. Increasing evidence suggests that selective mGluR2 (Corti et al., 2007; Fell et al., 2008; Rowe et al., 2008) activation may be a more effective target for pharmacotherapeutics of psychiatric disorders such as anxiety and schizophrenia. Amphetamine-induced hyperactivity was attenuated by mGluR2/3 agonists in mGluR3 knock out mice, but not in mGluR2 knock out mice (Fell et al., 2008; Woolley et al., 2008). Thus, the effects of LY379268 observed in the current studies may be mediated primarily via the mGluR2 receptor. Allosteric potentiators have been developed that selectively modulate the mGluR2 receptor. These selective potentiators may be more effective in attenuating the behavioral-stimulant and reinforcing effects of cocaine. Understanding the mechanisms by which LY379268 attenuates the effects of cocaine in nonhuman primates may be important for the development of pharmacotherapeutics for cocaine addiction.

A. Limitations

One of the primary limitations of the study was the limited pharmacological tools available with selective actions for either the mGluR2 or mGluR3. Thus, dissociation of the role of each of the two receptors was difficult. These receptors are differentially localized, mGluR2 predominantly on presynaptic neurons and mGluR3 predominantly

on astrocytes. This differential localization suggests that these receptors play different roles in normal neural activity. Furthermore, mGluR2 has been demonstrated to play a more significant role than mGluR3 in the underlying mechanisms of cocaine (Morishima et al., 2005; Woolley et al., 2008, 2007). The effects of LY379268 have been shown to be mediated by mGluR2 receptors and not mGluR3 receptors (Woolley et al., 2008, 2007). mGluR2 knock out mice show an enhanced responsiveness to cocaine (Morishima et al., 2005). Fortunately, there were systemically active agonists and antagonists selective for mGluR2/3 available. Future studies will benefit from the development of selective compounds to target mGluR2. Allosteric modulators are currently being developed and characterized and may be better pharmacotherapeutics for cocaine addiction (Benneyworth et al., 2007; Bonnefous et al., 2005; Cube et al., 2005; Galici et al., 2006; Govek et al., 2005; Johnson et al., 2003; Johnson et al., 2004; Pinkerton et al., 2005; Pinkerton et al., 2004a; Pinkerton et al., 2004b). Furthermore, data available for effective doses of glutamatergic drugs were derived from rodent studies. Thus, effective doses in nonhuman primates had to be established during the experiments. However, characterization of these drugs in vivo in nonhuman primates is important for establishing the generality of the effects of these compounds across species. Undoubtedly, development efforts will continue to generate and characterize appropriate pharmacological tools to manipulate mGluR function given the current therapeutic interests in glutamate. Additionally, the repeated measures design employed in this study resulted in individual subjects with extensive drug histories. To minimize this confound, drug doses were counterbalanced for individual test compounds and cocaine dose-effect curves were re-established before initiating experiments with different test compounds. Utilizing new groups of monkeys to control for drug history would be unrealistic due to cost as well as time required to train new groups.

B. Future Directions

Future studies will continue to emphasize the interactions of dopamine and glutamate. In the current studies, the focus was on the role of glutamatergic tone on mGlu2/3 receptors because of their localization within the VTA and nucleus accumbens (Kenny and Markou, 2004). mGluR5 receptors are also expressed within these areas (Kenny and Markou, 2004). Initial experiments would try to distinguish the role of mGluR2/3 from that of mGluR5 in the neurochemical and behavioral effects of cocaine. Though the behavioral-stimulant and reinforcing effects of cocaine are most attributed to dopamine, cocaine also binds to the norepinephrine transporter and serotonin transporter to enhance postsynaptic signaling of these monoamines. Conducting similar experiments as those proposed in these studies with a selective dopamine transporter inhibitor can better establish the interaction of glutamate with dopamine. The high probability of relapse is one of the hallmarks of cocaine addiction. Relapse can be induced by drug availability, environmental factors and stress. Extending these cocaine-induced reinstatement studies to cue- or stress-induced reinstatement would characterize the role of metabotropic glutamate receptors in other factors that may contribute to relapse. Future studies will also take advantage of better pharmacological tools to selectively manipulate mGluR2 and mGluR3. Undoubtedly, development efforts will continue to generate and characterize appropriate pharmacological tools to manipulate mGluR function given the current therapeutic interests in glutamate. Effective pharmacological treatment of cocaine abuse will necessitate repeated administration of a medication for an extended period, thus candidate compounds should have continued effectiveness during chronic treatment with minimal adverse effects. Lastly, future directions will begin to characterize the consequences of long-term stimulant use on glutamatergic function. Transitioning from recreational drug use to

abuse and dependence likely involves significant dysregulation of cortical glutamatergic activity.

In the current experiments, manipulations of the cystine-glutamate transporter were limited to the transporter enhancer, NAC. Future studies would benefit from the development of a systemic cystine-glutamate transporter blocker or from the design of behavioral experiments that could be conducted realistically with reverse dialysis. Given that glutamate is so highly regulated, measuring glutamate with techniques that allow for real-time measurement of extracellular glutamate will shed light on the interactions between dopamine and glutamate. Enzyme-immobilized biosensor technology is a relatively new methodology being developed that can be designed to measure real-time changes in extracellular glutamate and dopamine concurrently as specificity is based on the enzymes immobilized on the sensors. These sensors have been designed to be implanted utilizing microdialysis cannulae. Thus, the surgical protocols for implanting these sensors are well-established. These sensors have the added benefit of wireless telemetry and would allow for measuring both glutamate and dopamine during in vivo behavioral sessions with relative ease as compared to microdialysis. Once this technology is further developed and characterized, it can prove to be a valuable tool for measuring in vivo changes in neurochemistry. However, this technology would not replace the value of microdialysis, as it has the disadvantage of only measuring particular targets, while microdialysis allows for the measurement of a number of neurotransmitters at the same time. Nevertheless, as the same cannulae are utilized for both methods, both methods can be run in the same subjects.

C. Conclusion

The long-term goal of this project was to begin to characterize the interactions between glutamate and dopamine that may underlie the mechanisms of action of

cocaine. We utilized available pharmacological tools to characterize the role of mGluR2/3 receptors in cocaine-induced neurochemistry and behavior. The current studies provide the first evidence that pretreatment of NAC or LY379268 can attenuate the neurochemical effects of cocaine in nonhuman primates. The neurochemical effects of NAC were not reflected in changes in the behavioral pharmacology of cocaine, while the effects of LY379268, though consistent with previous studies, were only mildly reflected in changes in the behavioral pharmacology of cocaine. These studies suggest that the effects of NAC on behavior in nonhuman primates may be masked by other factors such as activation of multiple glutamate systems or activation of antioxidant systems. Though the effects of LY379268 administration in the behavioral pharmacology of cocaine were not as robust as in previously reported studies, these data provide further evidence for the need to develop better pharmacological tools to manipulate mGluR2/3 as potential targets for pharmacotherapeutics of cocaine addiction.

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