The Effects of Cocaine on the Dorsolateral Striatum of the Rat

by

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A dissertation

Presented to the Integrative Biomedical Sciences (IBMS) Program

And Oregon Health & Science University

School of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

April 2006

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

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Acknowledgments

Many people have helped me get to this point. First and foremost, I would like to thank my mentor, Charlie Meshul. Five and a half years ago I thought that he would make a great mentor, but I had no idea just how fortunate I would be. Charlie has been tremendously supportive and encourages me to pursue my passions. He fostered my creativity, guided and enhanced my scientific knowledge, and helped me to become a better person and scientist. We can talk for hours about science, or opera, or life, and I cherish our relationship.

I have had the fortunate experience of a helpful thesis committee. Steve Johnson has always made himself available to answer my pharmacology questions. He is a great scientist, who can consistently give a fresh perspective on an old question, and is also a wonderful clinician. Working in Sue Aicher's laboratory taught me the critical importance of good technique and about some of the fun things that can be done with immunohistochemistry. Sue's attention to detail and critical thinking has helped to direct my approach to research. Greg Mark has always been tremendously helpful with my microdialysis and HPLC questions. He personally trained me in the microinjection technique. His technical prowess is fantastic and he generously shares his expertise and supplies. I sincerely appreciate his consistent support. I asked Paul Berger to join the thesis exam committee because I admire his creativity and impressive knowledge of scientific literature.

The Meshul labmates, especially Cindy Moore, have made research fun and helped me in every aspect of lab life. The administrations of the Physiology/ Pharmacology and Behavioral Neuroscience departments have been lifesavers. Bill Cameron encouraged and supported me early in graduate school when I really needed it. Paul Meyer welcomed me and made me feel at home among the fabulous BEHN students, many of whom have become my friends. My IBMS friends have been my co-conspirators from the beginning, and I enjoyed "growing up" with them in graduate school. My family and friends have helped me walk through the ups and downs of graduate school. My sister and parents have been my cheerleaders from the very beginning and Samantha Johnson has helped me realize my potential. I would like to especially thank Kyle Ryan, who keeps me grounded. Their love and support makes all things possible.

Abbreviations

aCSF – artificial cerebral spinal fluid
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate
ANOVA – analysis of variance
cAMP – cyclic adenosine monophosphate
COC – cocaine
D1R – dopamine 1 receptor
D2R – dopamine 2 receptor
DAG – diacylglycerol
DAT – dopamine transporter
DLS – dorsolateral striatum
EAAC1 – excitatory amino acid carrier 1
EAAT – excitatory amino acid transporter
EGTA – ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPN - entopeduncular nucleus
GABA – gamma-aminobutyric acid
GFAP – glial fibrillary acidic protein
GLAST – glutamate/aspartate transporter
GLT-1 – glutamate transporter-1
GPe – external segment of the globus pallidus
GPi – internal segment of the globus pallidus
HPLC – high performance liquid chromatography
i.p. – intraperitoneal
IP_3 – inositol 1,4,5-triphosphate
KA – kainic Acid
mGluR – metabotropic glutamate receptors
mg/kg – milligram (of drug) per kilogram (of weight)
ml/kg – milliliter (of drug) per kilogram (of weight)
n – number of subjects
NAc – nucleus accumbens
NMDA – *N*-methyl-D-aspartate
PDC – L-*trans*-pyrrolidine-2,4-dicarboxylate
PKC – protein kinase C
s.c. – subcutaneous
S.E.M. – standard error of the mean
SNc – substantia nigra pars compacta
SNr – substantia nigra pars reticulate
STN – subthalamic nucleus
S-R – stimulus-response
TTX – tetrodotoxin
VEH – vehicle
VM – ventromedial nucleus of the thalamus, or motor thalamus
VMS – ventromedial striatum

Abstract

Acute cocaine administration has been shown to alter dorsal striatal plasticity (Graybiel et al., 1990;Torres and Rivier, 1993) and produce long-term neurochemical changes (Peris and Zahniser, 1987). However, the long-term effects on striatal glutamate after acute cocaine administration are not known. To investigate cocaine-induced changes in extracellular glutamate, *in vivo* microdialysis was carried out in the dorsolateral striatum of rats 1, 2, 3, and 14 days after receiving a single injection of either vehicle or 15 mg/kg cocaine. It was found that extracellular glutamate was increased 1 day after a single injection of cocaine in the rat dorsolateral striatum. Three days after a single cocaine injection, striatal glutamate decreased from control levels and this attenuation persisted 14 days later. The exact mechanism for this biphasic change in extracellular glutamate is unknown. These alterations in extracellular glutamate do not result from changes in vesicular glutamate within identified nerve terminals as measured by immunogold electron microscopy. The primary goal of this dissertation was to determine a possible mechanism for this biphasic change in striatal glutamate. It was hypothesized that the acute cocaine-induced changes in striatal glutamate were mediated by glutamate transporters and the thalamocortical input to the dorsolateral striatum. First, the role of the thalamocortical pathway and glutamate transporters was investigated in the 1-day increase in striatal glutamate. To determine whether cocaine increases extracellular glutamate by acting through the thalamocortical pathway, the motor thalamic nucleus was lesioned prior to acute cocaine exposure. Lesioning the motor thalamus eliminated the pathway that projects to the dorsolateral striatum through the cortex. The motor thalamic lesion blocked the increase in striatal glutamate found 1 day after a single cocaine exposure. Immunohistochemistry was used to determine the changes in the density of glutamate transporters in the dorsolateral striatum after acute cocaine exposure. Also, glial fibrillary acidic protein (GFAP) immunolabeling was used as a marker to determine if astrocyte proliferation could account for the reduction in

extracellular glutamate. The increase in striatal glutamate was associated with a decrease in striatal GFAP and GLAST immunolabeling 1 day after cocaine treatment. Next, the 3-day decrease in striatal glutamate was investigated. The decrease in striatal glutamate found 3 days after a single cocaine treatment was not associated with alterations in GFAP or glutamate transporter immunolabeling. To investigate the role of the glutamate transporter in the 3-day decrease in striatal glutamate, a glutamate transporter antagonist PDC was microinjected into the dorsolateral striatum prior to systemic cocaine administration. Inhibition of the glutamate transporters with PDC increased striatal glutamate in the cocaine-treated group as compared to the vehicle-treated group. These studies helped to elucidate the reason extracellular glutamate initially increased and then decreased in the dorsolateral striatum after a single injection of cocaine. A single injection of cocaine caused time-dependent biphasic alterations in striatal glutamate. Furthermore, these changes were associated with astrocyte and glutamate transporter alterations and were mediated by the thalamocortical input to the dorsolateral striatum.

General Introduction

Little is known about the effects of cocaine on the dorsolateral striatum because most studies have focused on the mesolimbic reward pathway. In addition, most studies focus on repeated or self-administered cocaine treatments. Acute studies are critical for isolating the effects of cocaine on the drug-naïve animal. Investigating the neurochemical and morphological effects of an initial dose of cocaine in the dorsolateral striatum is an important first step in learning about the changes in the striatum that could lead to a predisposition for continued drug use. The primary goal of this thesis was to investigate the effects of a single injection of cocaine in the dorsolateral striatum and how these changes are mediated. Specifically, the effects of a single injection of cocaine on extracellular glutamate, vesicular glutamate, glutamate transporters, and glial cells in the rat dorsolateral striatum were investigated. *In vivo* microdialysis was used to measure changes in extracellular glutamate and quantitative immunogold electron microscopy was used to determine changes in presynaptic nerve terminal glutamate labeling. Immunohistochemistry was used to quantify changes in glutamate transporter and GFAP immunolabeling. The overarching hypothesis was that a single systemic cocaine injection alters extracellular glutamate in the dorsolateral striatum and that these changes are mediated by the thalamocortical pathway and glutamate transporters. The introduction provides background on the dorsolateral striatum and the extended basal ganglia circuitry, the acute effects of cocaine, the sources of extracellular glutamate and how extracellular glutamate levels are maintained, and the relationship between striatal glutamate and dopamine.

Animal models of cocaine use

Many different paradigms have been used to study the effects of cocaine as a drug of abuse. These paradigms include acute and repeated cocaine injection models, operant responding for self-administered cocaine, intermittent injections designed to induce a specific

behavior, and injection paradigms that model 'binge' cocaine administration in humans. Each of these paradigms has its own value as a model for different stages of cocaine use. The research in this thesis focuses on the effects of a single cocaine injection in order to isolate the long-lasting changes that result from a single cocaine exposure. It has been suggested that the initial response to cocaine is critical for determining whether a person will be likely to take cocaine again (Volkow et al., 1999;Davidson et al., 1993). It is possible that the initial response to cocaine may promote a tendency to abuse the drug at a later time. Therefore, characterizing the neural changes that result from acute cocaine is important for understanding drug effects and predisposition to addiction (Volkow et al., 1999).

Studies on acute cocaine

Cocaine is a popular drug of abuse that blocks the dopamine transporter. Inhibition of dopamine reuptake leads to a rise in extracellular dopamine in the striatum (Hurd and Ungerstedt, 1989;Church et al., 1987). In addition, a single cocaine injection has long-lasting effects on extracellular dopamine. Peris and Zahniser tested how long a single cocaine injection would increase dopamine release in the dorsal striatum (Peris and Zahniser, 1987). Rats were injected with 10 mg/kg cocaine (i.p.) 1 day to 14 days prior to being analyzed for dopamine release *in vitro*. Slices of rat brains that contained the dorsal striatum were preloaded with [^{3}H]-dopamine (Peris and Zahniser, 1987). They used these slices to measure the amphetamine-induced tritium release after a single cocaine injection. They found that the amphetamine-induced release of [^{3}H]-dopamine was higher in rats injected with cocaine as compared to saline. The elevated [^{3}H]-dopamine release was present 1 day after the cocaine treatment, and was still augmented 14 days after a single cocaine injection. Taken together, these studies demonstrate that cocaine increases extracellular dopamine release long after the initial cocaine treatment.

Evidence for an effect of cocaine on glutamate within the dorsolateral striatum arises from immunohistochemical studies. It has been reported that an acute cocaine injection

induces striatal expression of c-Fos, an immediate early gene (Uslaner et al., 2001;Graybiel et al., 1990). In addition, an acute cocaine injection induced JunB and FRA (Fos-related antigen) immunoreactivites and FRA levels remained elevated 18 hours post-injection (Moratalla et al., 1996). Furthermore, there was a decrease in the cocaine-induced expression of c-Fos by prior administration of glutamate receptor antagonists such as ketamine (Torres and Rivier, 1993). This suggests that c-Fos induction by cocaine is partially mediated by glutamate in the dorsal striatum.

Reid and colleagues used *in vivo* microdialysis to determine if acute cocaine alters extracellular glutamate. They collected baseline samples from the striatum, and then injected the rats with saline, 15 mg/kg or 30 mg/kg i.p. cocaine. Their results show that extracellular glutamate levels do not change over a 2-hour time period in response to an acute injection of cocaine (Reid et al., 1997). However, the sampling occurred along both the dorsal and ventral aspects of the medial striatum, as opposed to the lateral striatum. Not only are the projections to the medial and lateral striatum different (Haber et al., 2000;McGeorge and Faull, 1989), but the role of these two striatal regions differ in behavioral tasks as well (Reading et al., 1991).

Is it possible, then, for a single injection of cocaine to alter extracellular glutamate? The ability of cocaine to increase striatal dopamine is dependent on glutamate (Moghaddam and Bolinao, 1994;Pap and Bradberry, 1995). Moghaddam and Bolinao used *in vivo* microdialysis to determine the role of glutamate on regulating extracellular dopamine levels in the striatum (Moghaddam and Bolinao, 1994). They found that cocaine increased extracellular dopamine when infused through the microdialysis probe. Interestingly, perfusion of the *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate (AMPA)/ kainic acid (KA) receptor antagonists reduced the cocaine-induced increase in striatal dopamine. This study raises the possibility that cocaine-induced increase in extracellular dopamine may be regulated by glutamate. Taken together, these studies suggest that extracellular glutamate may

not be immediately altered after a single injection of cocaine but instead may be altered later to regulate dopamine levels.

Circuitry of the basal ganglia

The basal ganglia are important for motor control. In particular, the dorsolateral striatum is the main output nucleus. The dorsolateral area of the caudate nucleus (striatum) differs from other striatal areas because it receives projections exclusively from the sensorimotor cortex, whereas the limbic and associative cortices project to other striatal areas (McGeorge and Faull, 1989). Since the dorsolateral striatum receives cortical projections almost exclusively from the sensorimotor cortex, the dorsolateral striatum is the rat homologue of the primate putamen. The dorsolateral striatum receives ipsilateral and contralateral projections from the sensorimotor cortex (McGeorge and Faull, 1989;Carman et al., 1965;Webster, 1961;Wilson, 1987) and activation of the corticostriatal pathway leads to an increase in extracellular glutamate in the dorsolateral striatum (Meshul et al., 1996).

The dorsolateral striatum receives dopaminergic input from the substantia nigra as well as the glutamatergic input from the sensorimotor cortex (Smith and Bolam, 1990;McGeorge and Faull, 1989;Betarbet et al., 1997) (Figure I1). These major inputs make glutamate and dopamine the main neurotransmitters in the dorsolateral striatum. Both the glutamatergic and dopaminergic afferents form synaptic contacts onto GABAergic medium spiny projection neurons, hence modulating output from the dorsolateral striatum. Corticostriatal afferents form asymmetrical (excitatory) contacts onto the head of spines, whereas nigral afferents form symmetrical (inhibitory) contacts onto the neck of the spines (Figure I2). The location of the synaptic contacts affects the input from the spine to the cell body and also makes the interactions between glutamate and dopamine important (Smith and Bolam, 1990).

Striatal GABAergic neurons project to the globus pallidus externus (GPe), the substantia nigra pars reticulata (SNr), and the entopeduncular nucleus, which is the rat homologue of the

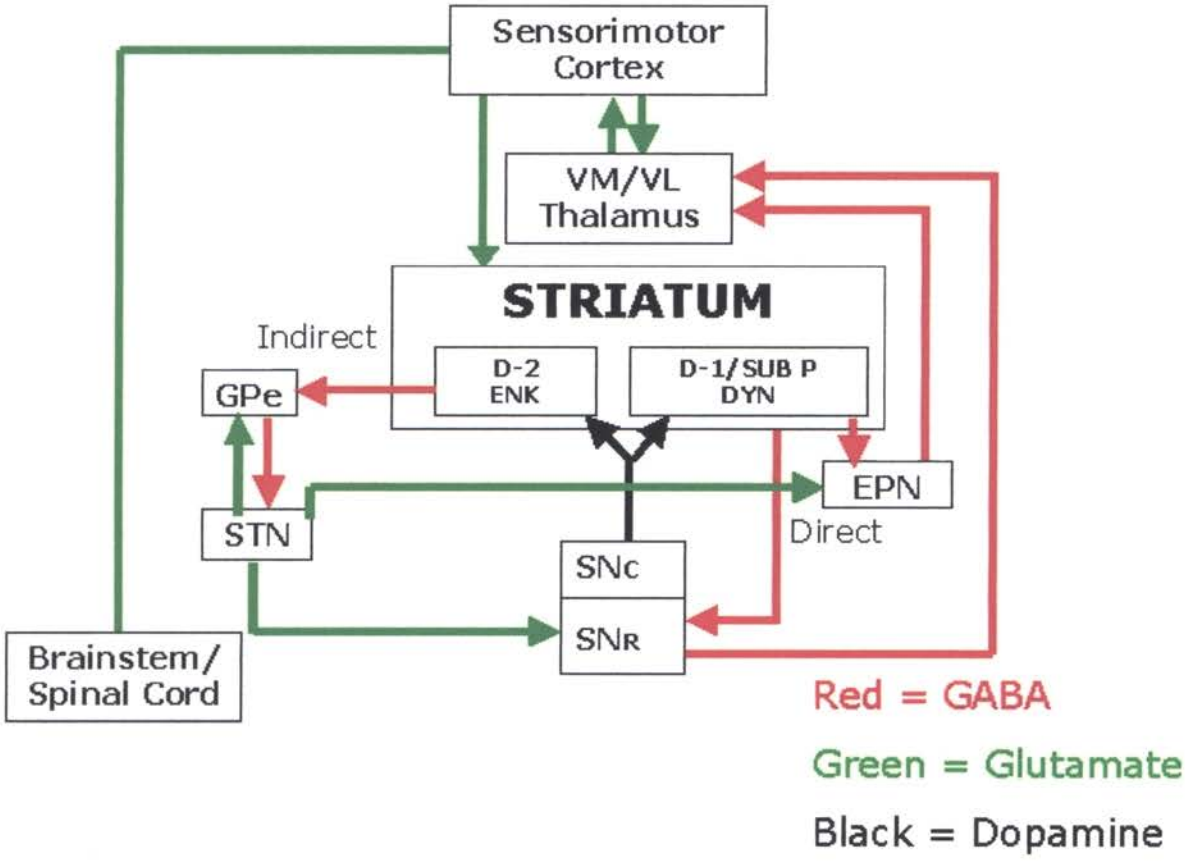

Figure I1: The major connections in the basal ganglia circuit. The red arrows represent GABAergic afferents, green arrows represent glutamatergic afferents, and the black arrow represents dopaminergic input. Abbreviations are D-1 (dopamine 1 receptors), D-2 (dopamine 2 receptors), Dyn (dynorphin), Enk (enkephalin), EPN (entopeduncular nucleus), GPe (globus pallidus externus), SNc (substantia nigra pars compacta), SNr (substantia nigra pars reticulata), STN (subthalamic nucleus), SUB P (substance P), and VM/VL (ventromedial/ ventrolateral nuclei of the thalamus). The direct pathway refers to the striatonigral/ striato-EPN projection and the indirect pathway refers to the striatopallidal projection.

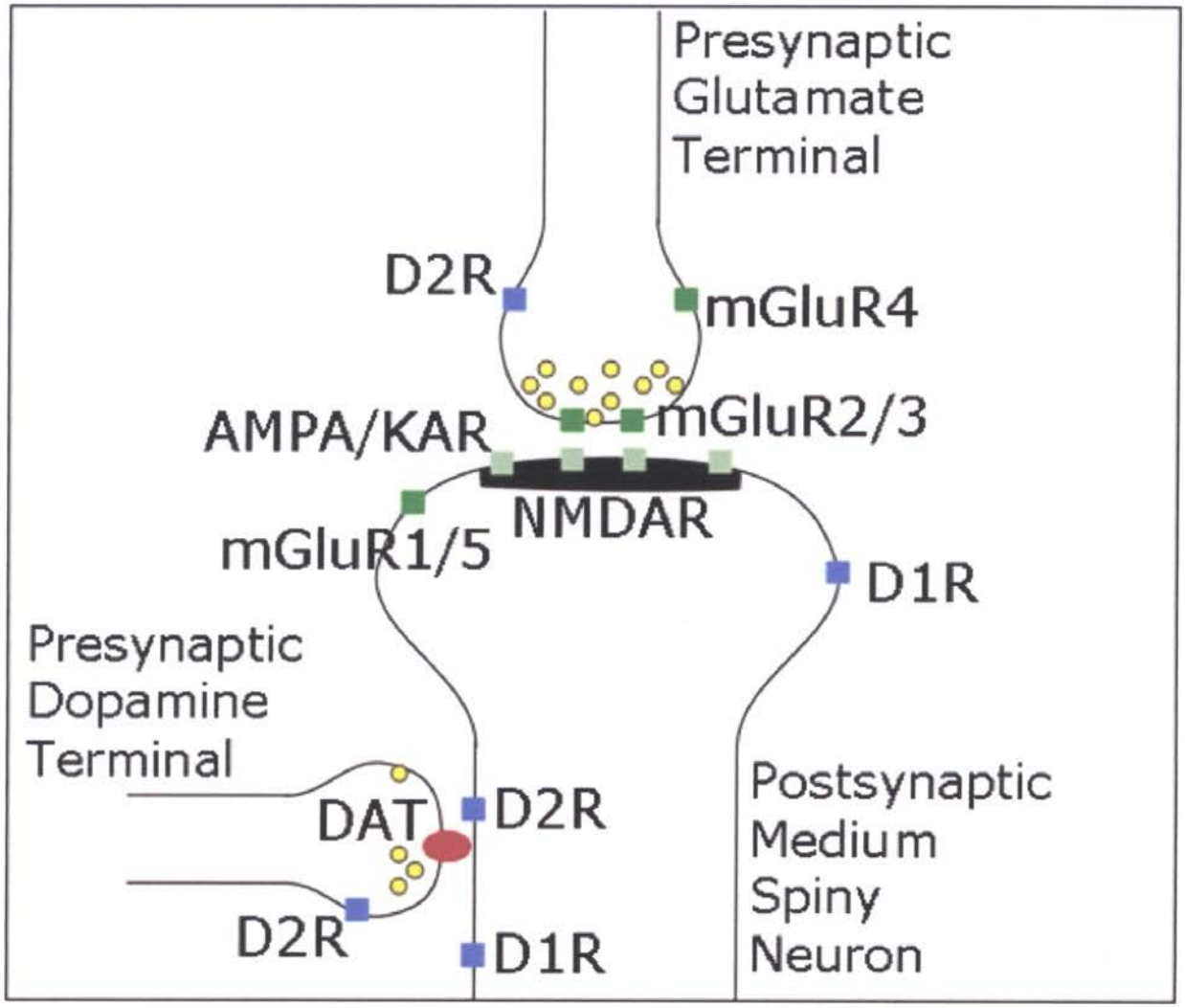

Figure I2: Cortical glutamate afferents and nigral dopamine afferents form asymmetrical and symmetrical synaptic contacts onto the spines of the medium spiny neuron in the striatum, respectively. The postsynaptic density on the head of the spine is represented as a black box to denote the formation of an asymmetrical (excitatory) synaptic contact. The abbreviations are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate) receptor, D1R (dopamine 1 receptor), D2R (dopamine 2 receptor), DAT (dopamine transporter), KA (kainic acid) receptor, mGluR – metabotropic glutamate receptors, and NMDA (*N*-methyl-D-aspartate) receptor. The yellow circles represent the vesicles within the nerve terminals.

globus pallidus internus. The direct pathway comprises the striatonigral and striato-EPN projections and the indirect pathway is composed of the striatopallidal projections (Wise et al., 1996). Controversial studies have suggested that the pathways have different types of postsynaptic dopamine receptors located on the striatal projection neurons (Yung et al., 1995;Gerfen et al., 1990). These data suggest that excitatory dopamine 1 receptors (D1R) are localized on the striatonigral neurons that project via the direct pathway, and that inhibitory dopamine 2 receptors (D2R) are localized to the striatopallidal neurons that project via the indirect pathway (Gerfen et al., 1990). However, electrophysiological and biochemical data suggest that there is colocalization of D1R and D2R on both striatopallidal and striatonigral efferents (Surmeier et al., 1996;Aizman et al., 2000). The segregation of dopamine receptors is important because when dopamine binds to D1R versus D2R, the striatal projection neuron is either activated or inhibited, respectively. When dopamine binds to D1Rs, adenylate cyclase activity is increased. This leads to an increase in the second messenger cyclic adenosine monophosphate (cAMP), which activates of the postsynaptic cell. However, D2R activation decreases adenylate cyclase activity, producing an inhibition of the postsynaptic cell. Activation of D1Rs on striatonigral/ striato-EPN neurons would increase GABA release in the SNr/EPN, and activation of the D2Rs on striatopallidal neurons would inhibit GABA release in the external segment of the globus pallidus (GPe).

The SNr/EPN sends an inhibitory GABAergic projection to the ventrolateral and ventromedial nuclei of the thalamus (VM/VL). The VM/VL nuclei of the thalamus comprise the motor thalamus because it is the VM/VL nuclei that project to the sensorimotor cortex. Projections to the motor thalamus originate from the GPe via the subthalamic nucleus (STN) and SNr. The motor thalamus sends a glutamatergic projection to the sensorimotor cortex and this thalamocortical loop projects back to the dorsolateral striatum. Direct thalamic input to the striatum exists, however this projection originates from the parafascicular nucleus (Sidibe et al., 2002). Parafascicular nucleus projections form synaptic contacts with dendritic shafts, not

spines (Smith and Bolam, 1990). Less than 10% of all striatal excitatory contacts are made onto shafts, suggesting that the thalamostriatal projection is minimal in the rat (Meshul et al., 1999). This implies that the motor thalamus does not have a direct influence on the dorsal striatum and that the main input to the dorsal striatum is indirect via the thalamocortical projection.

Glutamate/ dopamine interactions in the dorsolateral striatum

Cortical glutamate and nigral dopamine afferents form synaptic contacts onto the spines of medium spiny neurons in the striatum, and in this way can significantly impact the output from the striatum. Increased glutamate enhances spontaneous and evoked dopamine release (Kulagina et al., 2001;Moghaddam et al., 1990). Overall, increased glutamatergic activity would likely increase the output of the medium spiny neurons, thereby enhancing activity of the basal ganglia and the thalamocortical circuit. Attenuation of glutamatergic activity would likely reduce the activity of the basal ganglia. Conversely, dopamine can regulate cortical glutamate activity, presumably by binding to dopamine receptors located on corticostriatal terminals (Wang and Pickel, 2002;Cepeda et al., 1993;Cepeda et al., 2001). This possibility has been suggested by using a depolarizing concentration of potassium to increase extracellular glutamate in the striatum and then attenuating glutamate levels by perfusion of a D2R agonist through the microdialysis probe (Yamamoto and Davy, 1992). Another study showed that if glutamate concentration is above 10 μM in the rat striatum, then extracellular dopamine levels also increase (Moghaddam et al., 1990). However, it is probable that a 10 μM concentration of glutamate is attained only in pathophysiological conditions.

Sources of extracellular glutamate

The theories on the source of extracellular glutamate within the brain continue to be controversial (Timmerman and Westerink, 1997;Baker et al., 2002). The source of extracellular glutamate is of particular interest because *in vivo* microdialysis measures glutamate from the

extracellular space. Synaptic glutamate is not measured with *in vivo* microdialysis. The origin of extracellular glutamate is likely the summation of neuronal and glial sources. There are three ways that extracellular glutamate levels could be altered: changes in glutamate synthesis, glutamate release, or glutamate reuptake. Neuronal sources of glutamate include calcium-dependent release of glutamate, impulse-dependent release of glutamate, or both. Glial sources of extracellular glutamate include calcium-dependent release of glutamate from astrocytes, reuptake of glutamate into the astrocytes, the cystine/ glutamate antiporter, and reversal of the glutamate transporters located on astrocytes (Newman, 2003;Baker et al., 2002). In addition, the sources that contribute to striatal extracellular glutamate differ between the normal state and the cocaine-treated state (Rawls and McGinty, 1997).

Calcium-dependent release. Calcium-dependent release of glutamate has been measured by removing calcium (or significantly reducing calcium) from the aCSF perfused through the microdialysis probe. In addition, magnesium is added to the aCSF to restore the cation and chloride concentrations. Removing the calcium from the aCSF perfused through the microdialysis probe would reduce extracellular levels of glutamate if glutamate is released in a calcium-dependent manner. Studies have used the calcium removal method during microdialysis of the striatum and found a decrease in basal glutamate levels (Baker et al., 2002;Meshul et al., 2002;Wolf et al., 2000;Xue et al., 1996;Semba et al., 1995), no change in basal extracellular glutamate (McKee and Meshul, 2005;Timmerman and Westerink, 1997), or an increase in basal glutamate levels in anesthetized rats (Herrera-Marschitz et al., 1996). There are many variables that could contribute to these differences. In some studies, a calcium chelator is added to the aCSF like ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) to bind calcium. EGTA itself may cause glutamate release, which can alter basal glutamate levels (Herrera-Marschitz et al., 1997). Furthermore, placement of the microdialysis probe in the brain the day before the microdialysis experiment can diminish calcium sensitivity (Xue et al., 1996;Rawls and McGinty, 1997). Although there are mixed results on the calcium-

dependency of extracellular glutamate in the striatum, striatal extracellular glutamate levels do appear to be calcium-dependent after cocaine administration (McKee and Meshul, 2005;Smith et al., 1995).

Historically, calcium-dependency has been used to determine whether the source of extracellular glutamate is neuronal and released through vesicles (Herrera-Marschitz et al., 1997;Timmerman and Westerink, 1997;Nicholls and Attwell, 1990). However, data suggest that astrocytes can also release glutamate in a calcium-dependent manner (Montana et al., 2004;Newman, 2003;Nedergaard et al., 2003). Hence when calcium is removed from the aCSF that is perfused through the microdialysis probe, both the neuronal and glial release of glutamate may be affected.

Impulse-dependent release. Impulse-dependent release of glutamate has been measured by adding tetrodotoxin (TTX) into the aCSF that is perfused through the microdialysis probe. TTX blocks voltage-gated sodium channels and prevents neurons from generating an action potential. If extracellular glutamate is impulse-dependent, then perfusion of TTX would decrease extracellular glutamate after application. This method has been used to measure how much of neuronal release contributes to the levels of extracellular glutamate (Herrera-Marschitz et al., 1997). There are varying reports on the ability of TTX to reduce basal extracellular glutamate levels in the striatum (Timmerman and Westerink, 1997). This makes the use of TTX to measure impulse-dependent release of extracellular glutamate difficult. However, perfusion of a depolarizing concentration of potassium through the microdialysis probe has been shown to increase extracellular glutamate (Timmerman and Westerink, 1997;Herrera-Marschitz et al., 1997).

It has been suggested that the difficulty in measuring calcium-dependent and impulse-dependent release of glutamate is because the efficiency of the glutamate transporters masks the ability to detect changes in extracellular glutamate (Rawls and McGinty, 1997;Herrera-Marschitz et al., 1997). This hypothesis was tested by infusing glutamate transporter

antagonists into the striatum through the microdialysis probe. Infusion of glutamate transporter antagonists increased extracellular glutamate levels in the striatum (Rawls and McGinty, 1997;Herrera-Marschitz et al., 1996). The elevation of extracellular glutamate was attenuated by co-perfusion of aCSF containing either TTX or a low calcium concentration (Herrera-Marschitz et al., 1997;Rawls and McGinty, 1997). These data show that once glutamate transporters are unable to regulate extracellular levels of glutamate, the ability of calcium and neuronal activity to regulate extracellular glutamate becomes clear.

Sodium-independent release. Glutamate can be released through a cystine/ glutamate antiporter (Baker et al., 2002). The antiporter is a sodium- and calcium-independent transporter that exchanges extracellular cystine for intracellular glutamate (Baker et al., 2003). The antiporter is located on glial cells (Ye et al., 1999;Moran et al., 2003). Inhibition of the cystine/ glutamate antiporter reduces basal extracellular glutamate by 60% (Baker et al., 2002). It has been reported that infusion of the substrate cystine can return cocaine-induced attenuated glutamate levels to that of the control group in the nucleus accumbens (Baker et al., 2003). This suggests a role for the cystine/ glutamate antiporter in regulating extracellular glutamate levels in the cocaine-treated rat.

Functional significance of extracellular glutamate in the dorsolateral striatum

Regardless of the source of extracellular glutamate, extracellular glutamate drives the output from the dorsolateral striatum. Glutamate can bind to receptors and activate both the direct and indirect pathways, which constitutes the main output from the dorsolateral striatum. Therefore, glutamate is capable of mediating dorsolateral striatal behaviors. The functional significance of extracellular glutamate in the dorsolateral striatum is that glutamate is able to influence the following behaviors linked to the dorsolateral striatum: motor control (Graybiel, 1998), decision making and adaptive learning (Blazquez et al., 2002), drug-related behaviors such as stereotypy (Presti et al., 2003), procedural learning (Knowlton et al., 1996), and stimulus-response (habit) learning (Jog et al., 1999;Ito et al., 2002;Yin et al., 2004). In

particular, extracellular glutamate in the dorsolateral striatum may mediate stereotypic behaviors (Karler and Calder, 1992;Schmidt, 1986), and with repeated self-administration of psychostimulants, glutamate may be involved in stimulus-response (habit) learning (Packard, 1999).

Glutamate neurotransmission

Glutamate is released from neurons or astrocytes into the extracellular space. Synaptic glutamate can bind to postsynaptic glutamate receptors NMDA, AMPA/KA, and metabotropic glutamate receptors 1 and 5 (group I). NMDA and AMPA/KA receptors mediate the majority of the fast excitatory neurotransmission by opening cation channels that depolarize the postsynaptic cell. Metabotropic glutamate receptors (mGluR) 1 and 5 activate the postsynaptic cell by producing the inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) second messengers, which mobilize calcium release from the endoplasmic reticulum and activate protein kinase C (PKC), respectively. Metabotropic glutamate receptors 2 and 3 (group II) and 4 (group III) are located on the presynaptic terminal and differ by location on the nerve terminal (Figure I2) and also by the agonists and antagonists that bind to the receptors (Testa et al., 1994). Both mGluR group II and III inhibit adenylate cyclase activity and therefore decrease the cyclic adenosine monophosphate (cAMP) second messenger, leading to an inhibition of the presynaptic terminal.

Glutamate is also taken up by glutamate transporters and translocated into the postsynaptic cell or glial cells (Figure I3). Astrocytic glutamate is converted to glutamine via the enzyme glutamine synthetase (Rothstein and Tabakoff, 1984). Glutamine is not toxic and can be released into the extracellular space to be taken up into the nerve terminal. Once the glutamine is inside the nerve terminal, it is converted back into glutamate and can be packaged into vesicles for neuronal release. Inhibition of glutamate synthesis reduces glutamine in tissue by 50% and leads to a decrease in calcium-dependent,

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Sources of extracellular glutamate

The theories on the source of extracellular glutamate within the brain continue to be controversial (Timmerman and Westerink, 1997;Baker et al., 2002). The source of extracellular glutamate is of particular interest because *in vivo* microdialysis measures glutamate from the

extracellular space. Synaptic glutamate is not measured with *in vivo* microdialysis. The origin of extracellular glutamate is likely the summation of neuronal and glial sources. There are three ways that extracellular glutamate levels could be altered: changes in glutamate synthesis, glutamate release, or glutamate reuptake. Neuronal sources of glutamate include calcium-dependent release of glutamate, impulse-dependent release of glutamate, or both. Glial sources of extracellular glutamate include calcium-dependent release of glutamate from astrocytes, reuptake of glutamate into the astrocytes, the cystine/ glutamate antiporter, and reversal of the glutamate transporters located on astrocytes (Newman, 2003;Baker et al., 2002). In addition, the sources that contribute to striatal extracellular glutamate differ between the normal state and the cocaine-treated state (Rawls and McGinty, 1997).

Calcium-dependent release. Calcium-dependent release of glutamate has been measured by removing calcium (or significantly reducing calcium) from the aCSF perfused through the microdialysis probe. In addition, magnesium is added to the aCSF to restore the cation and chloride concentrations. Removing the calcium from the aCSF perfused through the microdialysis probe would reduce extracellular levels of glutamate if glutamate is released in a calcium-dependent manner. Studies have used the calcium removal method during microdialysis of the striatum and found a decrease in basal glutamate levels (Baker et al., 2002;Meshul et al., 2002;Wolf et al., 2000;Xue et al., 1996;Semba et al., 1995), no change in basal extracellular glutamate (McKee and Meshul, 2005;Timmerman and Westerink, 1997), or an increase in basal glutamate levels in anesthetized rats (Herrera-Marschitz et al., 1996). There are many variables that could contribute to these differences. In some studies, a calcium chelator is added to the aCSF like ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) to bind calcium. EGTA itself may cause glutamate release, which can alter basal glutamate levels (Herrera-Marschitz et al., 1997). Furthermore, placement of the microdialysis probe in the brain the day before the microdialysis experiment can diminish calcium sensitivity (Xue et al., 1996;Rawls and McGinty, 1997). Although there are mixed results on the calcium-

dependency of extracellular glutamate in the striatum, striatal extracellular glutamate levels do appear to be calcium-dependent after cocaine administration (McKee and Meshul, 2005;Smith et al., 1995).

Historically, calcium-dependency has been used to determine whether the source of extracellular glutamate is neuronal and released through vesicles (Herrera-Marschitz et al., 1997;Timmerman and Westerink, 1997;Nicholls and Attwell, 1990). However, data suggest that astrocytes can also release glutamate in a calcium-dependent manner (Montana et al., 2004;Newman, 2003;Nedergaard et al., 2003). Hence when calcium is removed from the aCSF that is perfused through the microdialysis probe, both the neuronal and glial release of glutamate may be affected.

Impulse-dependent release. Impulse-dependent release of glutamate has been measured by adding tetrodotoxin (TTX) into the aCSF that is perfused through the microdialysis probe. TTX blocks voltage-gated sodium channels and prevents neurons from generating an action potential. If extracellular glutamate is impulse-dependent, then perfusion of TTX would decrease extracellular glutamate after application. This method has been used to measure how much of neuronal release contributes to the levels of extracellular glutamate (Herrera-Marschitz et al., 1997). There are varying reports on the ability of TTX to reduce basal extracellular glutamate levels in the striatum (Timmerman and Westerink, 1997). This makes the use of TTX to measure impulse-dependent release of extracellular glutamate difficult. However, perfusion of a depolarizing concentration of potassium through the microdialysis probe has been shown to increase extracellular glutamate (Timmerman and Westerink, 1997;Herrera-Marschitz et al., 1997).

It has been suggested that the difficulty in measuring calcium-dependent and impulse-dependent release of glutamate is because the efficiency of the glutamate transporters masks the ability to detect changes in extracellular glutamate (Rawls and McGinty, 1997;Herrera-Marschitz et al., 1997). This hypothesis was tested by infusing glutamate transporter

antagonists into the striatum through the microdialysis probe. Infusion of glutamate transporter antagonists increased extracellular glutamate levels in the striatum (Rawls and McGinty, 1997;Herrera-Marschitz et al., 1996). The elevation of extracellular glutamate was attenuated by co-perfusion of aCSF containing either TTX or a low calcium concentration (Herrera-Marschitz et al., 1997;Rawls and McGinty, 1997). These data show that once glutamate transporters are unable to regulate extracellular levels of glutamate, the ability of calcium and neuronal activity to regulate extracellular glutamate becomes clear.

Sodium-independent release. Glutamate can be released through a cystine/ glutamate antiporter (Baker et al., 2002). The antiporter is a sodium- and calcium-independent transporter that exchanges extracellular cystine for intracellular glutamate (Baker et al., 2003). The antiporter is located on glial cells (Ye et al., 1999;Moran et al., 2003). Inhibition of the cystine/ glutamate antiporter reduces basal extracellular glutamate by 60% (Baker et al., 2002). It has been reported that infusion of the substrate cystine can return cocaine-induced attenuated glutamate levels to that of the control group in the nucleus accumbens (Baker et al., 2003). This suggests a role for the cystine/ glutamate antiporter in regulating extracellular glutamate levels in the cocaine-treated rat.

Functional significance of extracellular glutamate in the dorsolateral striatum

Regardless of the source of extracellular glutamate, extracellular glutamate drives the output from the dorsolateral striatum. Glutamate can bind to receptors and activate both the direct and indirect pathways, which constitutes the main output from the dorsolateral striatum. Therefore, glutamate is capable of mediating dorsolateral striatal behaviors. The functional significance of extracellular glutamate in the dorsolateral striatum is that glutamate is able to influence the following behaviors linked to the dorsolateral striatum: motor control (Graybiel, 1998), decision making and adaptive learning (Blazquez et al., 2002), drug-related behaviors such as stereotypy (Presti et al., 2003), procedural learning (Knowlton et al., 1996), and stimulus-response (habit) learning (Jog et al., 1999;Ito et al., 2002;Yin et al., 2004). In

particular, extracellular glutamate in the dorsolateral striatum may mediate stereotypic behaviors (Karler and Calder, 1992;Schmidt, 1986), and with repeated self-administration of psychostimulants, glutamate may be involved in stimulus-response (habit) learning (Packard, 1999).

Glutamate neurotransmission

Glutamate is released from neurons or astrocytes into the extracellular space. Synaptic glutamate can bind to postsynaptic glutamate receptors NMDA, AMPA/KA, and metabotropic glutamate receptors 1 and 5 (group I). NMDA and AMPA/KA receptors mediate the majority of the fast excitatory neurotransmission by opening cation channels that depolarize the postsynaptic cell. Metabotropic glutamate receptors (mGluR) 1 and 5 activate the postsynaptic cell by producing the inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) second messengers, which mobilize calcium release from the endoplasmic reticulum and activate protein kinase C (PKC), respectively. Metabotropic glutamate receptors 2 and 3 (group II) and 4 (group III) are located on the presynaptic terminal and differ by location on the nerve terminal (Figure I2) and also by the agonists and antagonists that bind to the receptors (Testa et al., 1994). Both mGluR group II and III inhibit adenylate cyclase activity and therefore decrease the cyclic adenosine monophosphate (cAMP) second messenger, leading to an inhibition of the presynaptic terminal.

Glutamate is also taken up by glutamate transporters and translocated into the postsynaptic cell or glial cells (Figure I3). Astrocytic glutamate is converted to glutamine via the enzyme glutamine synthetase (Rothstein and Tabakoff, 1984). Glutamine is not toxic and can be released into the extracellular space to be taken up into the nerve terminal. Once the glutamine is inside the nerve terminal, it is converted back into glutamate and can be packaged into vesicles for neuronal release. Inhibition of glutamate synthesis reduces glutamine in tissue by 50% and leads to a decrease in calcium-dependent,

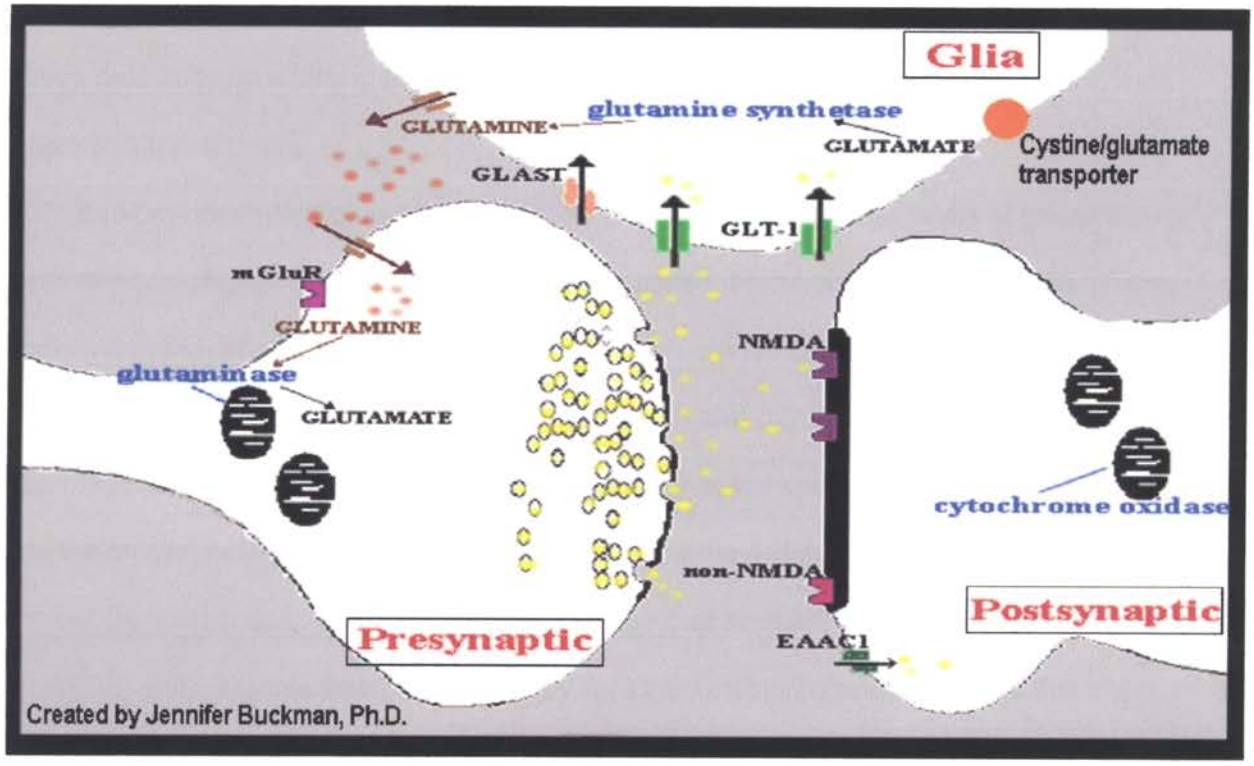


Figure I3: Glutamate neurotransmission involves the release of vesicular glutamate (yellow circles) into the synaptic cleft, where glutamate can bind to glutamate receptors. Uptake of glutamate from the transporters occurs at postsynaptic cells (EAAC1) and glial cells (GLT-1 and GLAST). Glutamate can also be released into the extracellular space by the cystine/ glutamate transporter.

potassium-stimulated release of glutamate from nerve terminals (Rothstein and Tabakoff, 1984). These data suggest a strong relationship between glial and neuronal glutamate.

Glutamate transporters

Sodium-dependent glutamate transporters regulate extracellular levels of glutamate by rapid removal of glutamate from the extracellular space (Nicholls and Attwell, 1990). Three sodium-dependent glutamate transporters have been cloned in the rodent and were originally named excitatory amino acid transporter (EAAT) 1, 2, and 3 (Rothstein et al., 1994). Glutamate/ aspartate transporter (GLAST or EAAT1) and glutamate transporter-1 (GLT-1 or EAAT2) are located on glial cells and the excitatory amino acid carrier 1 (EAAC1 or EAAT3) transporter is located on postsynaptic neurons (Figure I3)(Haugeto et al., 1996). The exclusive localization of GLAST to glial cells has been questioned by light immunohistochemical studies that show GLAST may be present in both glia and neurons (Rothstein et al., 1994).

GLT-1 and GLAST regulate extracellular glutamate levels by reuptake of glutamate from the extracellular space into astroglia. It has been reported that inhibiting GLAST and GLT-1 with specific antisense deoxyoligonucleotides increases extracellular glutamate (Rothstein et al., 1996). EAAC1 regulates extracellular glutamate levels by reuptake of glutamate from the extracellular space into the postsynaptic spines. However, extracellular glutamate is not increased by inhibition of EAAC1 (Rothstein et al., 1994).

GLT-1 has been suggested as the predominant glutamate transporter for the striatum (Rothstein et al., 1994). Inhibiting the synthesis of the different transporters in the striatum with specific antisense deoxyoligonucleotides allowed the role of each transporter in glutamate reuptake to be measured (Rothstein et al., 1996). Loss of the glial glutamate transporters GLT-1 and GLAST reduced glutamate reuptake by 56% and 35%, respectively. Loss of striatal EAAC1 reduced functional glutamate transport by 22%. Immunoblots of striatal tissue homogenates show that a large amount of the GLT-1 protein, a small amount of EAAC1 protein, and very little GLAST (Rothstein et al., 1994). However, immunoreactivity of GLAST was higher

in the striatum than the immunoblots suggested (Rothstein et al., 1994). Furthermore, ultrastructure of the striatum showed GLAST-positive astroglial processes (Rothstein et al., 1994). These studies suggest that the glial glutamate transporters take up the majority of glutamate in the extracellular space.

Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is a marker that has been used to detect changes in mature astrocyte proliferation (Buckman and Meshul, 1999;Bowers and Kalivas, 2003). GFAP is a structural filament specific to mature astrocytes (Bowers and Kalivas, 2003). Because glial glutamate transporters are located on astrocytes, changes in the density of GFAP immunolabeling might alter GLT-1 and GLAST immunolabeling. For example, a proliferation in astrocytes could increase the number of glutamate transporters available to remove glutamate from the synaptic cleft. This would lead to an increase in density of glial glutamate transporter immunolabeling.

Rationale

The summarized literature suggests that acute cocaine may have long-lasting effects on the glutamate system in the dorsolateral striatum. The main goal of this thesis research was to determine what neurochemical and morphological changes occur in the dorsolateral striatum after acute cocaine administration and how these changes might be mediated. The overall hypothesis was that acute cocaine-induced changes in striatal glutamate are mediated by the thalamocortical pathway and glutamate transporters.

The first set of experiments in chapter 1 determined the effects of an acute injection of cocaine on glutamate in the dorsolateral striatum of rats. These studies examined the changes in extracellular and presynaptic glutamate in the dorsal striatum after a single injection of cocaine 1, 2, 3, and 14 days prior to performing *in vivo* microdialysis. Immunogold electron microscopy was used to show changes in presynaptic glutamate immunolabeling in the dorsolateral striatum. The working hypothesis was that extracellular glutamate would increase

and the relative density of glutamate immunolabeling in striatal nerve terminals would decrease as a result a single injection of cocaine. It was expected that a single injection of cocaine would increase striatal glutamate through the basal ganglia circuitry. Furthermore, this increase would be inversely related to a decrease in glutamate immunolabeling within corticostriatal nerve terminals (i.e. less glutamate within the nerve terminals because increased extracellular glutamate).

The experiments in chapter 2 determined the effects of a motor thalamic lesion on extracellular glutamate in the dorsolateral striatum after acute cocaine administration. To investigate the role of the thalamocortical pathway in cocaine-induced alterations of striatal glutamate, changes in extracellular glutamate were measured in thalamic and sham-lesioned animals 1 day after acute cocaine or vehicle treatment. The motor thalamus was lesioned to show the role of the thalamocortical pathway in the cocaine-induced increase in striatal glutamate because the dorsolateral striatum receives a direct input from the thalamocortical projection. In addition, the effects of a single cocaine injection on GFAP and glutamate transporter GLT-1, GLAST, and EAAC1 immunolabeling were measured 1 day after the acute cocaine exposure. The working hypothesis was that a lesion of the motor thalamus would block the cocaine-induced increase in striatal glutamate levels 1 day after cocaine treatment. It was also hypothesized that the relative density of glutamate transporter immunolabeling would decrease 1 day after cocaine treatment.

The experiments in chapter 3 addressed the contribution of the glutamate transporters to the cocaine-induced attenuation in extracellular glutamate found 3 days after a single cocaine injection. Rats received an intrastriatal microinjection of a glutamate transporter antagonist prior to an intraperitoneal injection of cocaine. Microdialysis was carried out 3 days after receiving these injections to determine if glutamate transporter inhibition would block the cocaine-induced decrease in striatal glutamate. In addition, changes in GFAP and glutamate transporter GLT-1, GLAST, and EAAC1 immunolabeling were assessed to determine whether a single injection of

cocaine alters astrocytes and glutamate transporters 3 days later. The working hypothesis was that antagonism of the glutamate transporter would block the cocaine-induced decrease in striatal glutamate found 3 days after a single cocaine injection. It was further hypothesized that cocaine produces a decrease in extracellular glutamate by astrocyte proliferation and increased expression of glutamate transporters.

The research in this dissertation sought to characterize changes in extracellular glutamate in the dorsolateral striatum resulting from a single cocaine injection. The additional experiments in chapters 2 and 3 provide evidence of potential mechanisms for the cocaine-induced changes in striatal glutamate. These experiments investigate the role of the motor thalamus, calcium, presynaptic glutamate, GFAP, and the glutamate transporters in the cocaine-induced changes in striatal glutamate. A single injection of cocaine caused long lasting, biphasic changes in extracellular glutamate in the rat dorsolateral striatum. Both neuronal and glial mechanisms contribute to the time-dependent changes in striatal glutamate following a single cocaine exposure. Characterizing the neural changes that result from a single injection of cocaine is important for understanding behavioral effects and predisposition to addiction (Volkow et al., 1999). The long lasting effects of an initial exposure to cocaine in the dorsolateral striatum may be important for predisposition to the development of habit formation (Canales, 2005).

Chapter 1

Time-dependent changes in extracellular glutamate in the rat dorsolateral striatum following a single cocaine injection

Abstract

Acute cocaine administration has been shown to alter dorsal striatal plasticity (Graybiel et al., 1990;Torres and Rivier, 1993) and produce long-term neurochemical changes (Peris and Zahniser, 1987). To date, the effects of acute cocaine on extracellular glutamate and nerve terminal glutamate immunolabeling in the rat dorsolateral striatum have not been reported. To investigate cocaine-induced changes in extracellular glutamate, *in vivo* microdialysis was carried out in the dorsolateral striatum of rats 1 to 14 days after receiving a single injection of either vehicle or 15 mg/kg cocaine. There was an increase in the group injected with cocaine 1 day prior to measuring extracellular glutamate as compared to the control group. The group injected with cocaine 3 days prior to the microdialysis session had decreased extracellular glutamate levels. Furthermore, extracellular glutamate remained attenuated 14 days after acute cocaine treatment. Striatal glutamate decreased in the cocaine-treated rats after calcium removal, suggesting that cocaine-induced changes in extracellular glutamate were partially calcium-dependent. The density of nerve terminal glutamate immunolabeling was measured using immunogold electron microscopy in the contralateral striatum of the same rats that had been acutely treated with cocaine or vehicle. There were no changes in the density of glutamate immunolabeling within identified nerve terminals making an asymmetrical (excitatory) synaptic contact 1, 2, 3, or 14 days after acute cocaine exposure as compared to the control groups. Hence, these alterations in extracellular glutamate did not result from changes in glutamate immunolabeling within the synaptic vesicle pool. In addition, no changes in glutamate immunolabeling were found in rats that received cocaine 2 hours previously or were withdrawn after 1 week of cocaine administration. The results demonstrate that a single injection of cocaine produces biphasic, time-dependent changes in extracellular glutamate in the rat dorsolateral striatum.

Introduction

Cocaine is a popular drug of abuse that blocks the reuptake of dopamine. The subsequent rise in extracellular dopamine results in behavioral changes, including stereotypy and locomotor stimulation (Robinson and Becker, 1986). A single cocaine exposure is sufficient to produce long-lasting behavioral and neurochemical sensitization in the rat (Peris and Zahniser, 1987;Robinson and Becker, 1986;Guan et al., 1985).

The dorsolateral striatum receives dopaminergic inputs from the substantia nigra and glutamatergic afferents originating from the sensorimotor cortex (McGeorge and Faull, 1989). These afferents make synaptic contacts with GABAergic medium spiny projection neurons, hence modulating output from the dorsolateral striatum. Glutamate drives the output signal from the dorsolateral striatum, whereas dopamine modulates the activation of the medium spiny neuron. Elevated extracellular dopamine may alter cortical glutamate activity by binding to dopamine D-2 receptors located on corticostriatal terminals (Sesack et al., 1994;Bamford et al., 2004b;Bamford et al., 2004a;Cepeda et al., 2001). Dopamine could also increase extracellular glutamate by activating the striato-GPi/SNr pathway and thereby increase corticostriatal activity. Hence, the interactions between dopamine and glutamate are critical for the output of the dorsal striatum, which regulates movement and behavior.

The dorsal striatum has been implicated in a variety of cocaine-related behaviors including locomotion, stereotypy, and behavioral sensitization (Robinson and Becker, 1986;Karler et al., 1995;Karler and Calder, 1992;Canales and Graybiel, 2000). Although most studies have focused on the role of dopamine in cocaine-related behaviors, glutamate influences these behaviors as well. NMDA antagonists block acute cocaine-stimulated behaviors, and therefore it is likely that the effects of cocaine are at least partially mediated by glutamate (Karler and Calder, 1992;Mao and Wang, 2000).

Evidence for an acute effect of cocaine within the dorsal striatum arises from neurochemical and immunohistochemical studies. Release of ^{3}H –dopamine from striatal slices increased 1 day after a single cocaine injection and remained increased 2 weeks later (Peris

and Zahniser, 1987). Acute cocaine increases extracellular dopamine in the dorsal striatum (Church et al., 1987;Hurd and Ungerstedt, 1989). A single injection of cocaine induced striatal expression of c-fos, an immediate early gene (Uslaner et al., 2001;Graybiel et al., 1990). This cocaine-induced expression of c-fos was diminished by prior administration of glutamate receptor antagonists such as ketamine (Torres and Rivier, 1993). This suggests that c-fos induction by acute cocaine is partially mediated by glutamate receptors in the dorsal striatum. In addition, acute cocaine treatment induced JunB and FRA (Fos-related antigen) expression in the dorsal striatum (Moratalla et al., 1996). Interestingly, FRA immunoreactivity was long lasting and remained elevated 18 hours after the acute cocaine exposure.

Using quantitative immunogold electron microscopy, we have reported that the density of glutamate immunolabeling associated with the synaptic vesicle pool within nerve terminals making an asymmetrical (excitatory) synaptic contact was decreased three days after a single injection of cocaine in the nucleus accumbens core and shell (Kozell and Meshul, 2003;Kozell and Meshul, 2001). Decreased glutamate immunolabeling suggests that there was less glutamate in the nerve terminals. This reduction could arise from an increase in glutamate release, a decrease in glutamate synthesis, or less glutamate reuptake into the terminals. Interestingly, in the nucleus accumbens core, this decrease was maintained for longer than 2 weeks after the original cocaine injection (Kozell and Meshul, 2003). Collectively, these studies raise the possibility that a single dose of cocaine may change glutamate within the dorsal striatum and these changes may be long term as previously shown in the ventral striatum (i.e. nucleus accumbens).

To investigate whether a single injection of cocaine alters glutamate in the dorsolateral striatum, *in vivo* microdialysis and quantitative immunogold electron microscopy were used to quantify alterations in striatal glutamate in rats previously exposed to an acute injection of cocaine. It was found that basal extracellular glutamate in the dorsolateral striatum increased in rats exposed to cocaine 1 day prior to the microdialysis session and decreased in rats injected

with cocaine 3 days prior to the experiment as compared to controls. Striatal glutamate remained reduced 14 days after a single cocaine exposure. There was no change in the density of presynaptic nerve terminal glutamate immunolabeling in rats that had previously received a single or repeated cocaine injection as compared to the control group. These data suggest that there are time-dependent alterations in striatal extracellular glutamate following a single cocaine injection.

Methods

Subjects

Male Sprague-Dawley rats (3 months old, Harlan, Indianapolis, IN) were housed at the Veterinary Medical Unit at the Veteran's Administration Medical Center in Portland, Oregon. Pairs of animals were housed in clear plastic cages with corncob bedding. Animals were maintained in a temperature and light controlled room on a 12-hour light/ dark cycle, with access to food and water ad libitum. All animals were drug and experimentally naïve at the beginning of the experiments. Care was taken to minimize the number of animals used and to reduce animal discomfort. Whenever possible, the same animal was used for both in vivo microdialysis and immunogold electron microscopy to reduce the number of animals needed in the experiments. All procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Veteran's Affairs Medical Center's Institutional Animal Care and Use Committee (IACUC).

Drugs

In all experiments, rats were administered cocaine hydrochloride dissolved in sterile water or 0.2 ml sterile water for vehicle-treated animals. In the first experiment, separate groups

of rats were injected with either vehicle or 15 mg/kg cocaine intraperitoneally. Vehicle or cocaine-treated rats were injected and in vivo microdialysis and glutamate immunogold electron microscopy were carried out 1, 2, 3, and 14 days post-injection. A rat was used for a single time point, and each cocaine-treated group had its own vehicle-treated group. In the next experiments, rats were injected with vehicle, 15 mg/kg cocaine, or 30 mg/kg cocaine subcutaneously. Extracellular glutamate and nerve terminal glutamate immunolabeling were quantified in each group. In the final experiment, rats received subcutaneous vehicle or 15 mg/kg cocaine injections for 7 days. The animals were withdrawn for 14 days and nerve terminal glutamate immunolabeling was measured in these animals.

Electron Microscopy

The rats were deeply anesthetized with a 5% ketamine, 2% xylazine, and 1% acepromazine (1 ml/kg, i.p.). Animals were transcardially perfused initially with 6 ml of heparin (1000 units/ ml in 0.1M HEPES buffer) and then with 300 ml of fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.1% picric acid in 0.1 M HEPES buffer, pH 7.3) using a peristaltic pump (Cole Parmer, Vernon Hills, IL)(Paxinos and Watson, 2005). The brains were promptly removed and placed in fixative overnight at 4°C. The brains were washed in cold 0.1M HEPES buffer the next morning. Brains were washed daily with cold 0.1M HEPES buffer until they were dissected.

The tissue from each experiment was cut and processed on the same day to eliminate variables resulting from dissecting and processing. The rostral dorsolateral caudate was dissected into 1x1x2 mm pieces (mm from Bregma: +1.6 to +0.5 mm rostral, -2.8 to –3.8 mm lateral, and –3.4 to –4.8 mm ventral). The tissue was then incubated in 1% osmium tetraoxide/ 1.5% potassium ferricyanide solution for 30 minutes. The tissue was washed in sterile water, and then immersed in aqueous 0.5% uranyl acetate for 30 minutes in the dark. The tissue was washed in sterile water and then dehydrated with increasing concentrations of alcohol. Tissue

was then placed in propylene oxide and incubated in a 1:1 mixture of Epon/Spurr resin and propylene oxide overnight. The next day, tissue was immersed in a 100% solution of resin, and then embedded in the Epon/Spurr resin at 60°C. All tissue processing steps were performed at room temperature except for the final embedding step.

Immunocytochemistry

Post-embedding immunogold electron microscopy was carried out as reported (Phend et al., 1992) with modifications (Meshul et al., 1994). Tissue embedded in plastic resin was thin-sectioned (90 nm) using an RMC ultramicrotome MT6000 (Tucson, AZ) and the section was placed onto 200 mesh nickel grids covered with a Coat-Quik G pen solution (Kiyota International; Elk Grove Village, IL). The sections were dried for at least 3 hours, and washed in a Tris buffer with Triton X-100 (TBST, pH 7.6) (0.5M Tris in 0.9% NaCl and 0.1% Triton X-100). The grids were then incubated overnight in a solution containing the primary antibody (1:10,000) against glutamate (polyclonal rabbit IgG, Sigma Chem. Co., St. Louis, MO) containing aspartate (1 mM) to prevent any cross-reactivity with this amino acid. The grids were incubated in a moist chamber to prevent evaporation of the antibody solution. The glutamate antibody has been characterized and the specificity of the antibody has been established (Meshul et al., 1994;Hepler et al., 1988).

The next day, the tissue was washed twice (5 minutes each) in the TBST pH 7.6 solution, and then washed again for 30 minutes. Then the grids were washed for 5 minutes in a TBST solution with a pH of 8.2. The tissue was incubated with the secondary antibody (goat anti rabbit IgG) conjugated to a 10-nm gold particle (1:50 dilution in TBST pH 8.2) (Amersham, Arlington Heights, IL). The grids were rinsed in deionized water and then counterstained with uranyl acetate and lead citrate using a Leica Microsystems Ultrastainer (Germany). The grids were dried and viewed with a transmission electron microscope (JEOL 1200 EX TEMSCAN) at 40,000x magnification. Ten photomicrographs per animal were obtained randomly throughout

the striatal neuropil (area containing the highest number of synapses). The images were captured with a digital AMT camera (Danvers, MA).

Glutamatergic nerve terminals were identified by the formation of an asymmetrical synaptic contact with a dendritic spine and by the accumulation of at least three synaptic vesicles within the nerve terminal. All nerve terminals forming a synaptic contact with a spine containing a postsynaptic density were counted in the analysis. The particle density of gold labeling associated with the synaptic vesicle pool in a nerve terminal was analyzed using an imaging program (ImagePro, Media Cybernetics, Silver Spring, MD). The experimenter was blinded to the specific treatment groups. The density (# particles/ μm^2) of glutamate immunogold labeling in identified nerve terminals making an asymmetrical contact onto dendritic spines was quantified. The metabolic pool of glutamate (i.e. glutamate immunolabeling associated with mitochondria) was eliminated from the density calculations.

Cannula Implantation

Anesthetized animals had their heads shaved and were placed into the Stoelting stereotaxic apparatus. The skin above their skulls was cut and their skulls were exposed. A small hole was drilled using a dental drill at the following coordinates: (mm from Bregma) +0.5 rostral and +3.1 lateral for cannula placement above the dorsolateral striatum (Paxinos and Watson, 2005). Three additional holes were drilled and dental screws were placed in the holes. A stainless steel guide cannula (15 mm long, 20-gauge) (Small Parts, Miami Lakes, FL) was lowered 0.5 mm from the surface of the skull without penetrating the dura. Dental cement was applied to the base of the guide cannula and the three flathead screws to stabilize the guide cannula assembly. Epoxy was added on top of the dental cement to further stabilize the assembly. Then the skin around the assembly is stapled and the rats were allowed to recover for at least five days.

In Vivo Microdialysis

Dialysis probes were assembled according to Robinson and Wishaw (Robinson and Whishaw, 1988) with modifications (Meshul et al., 1999). The probes were 210 μm in diameter and the cellulose tips were 2 mm in length to effectively target the dorsolateral quadrant of the striatum. The day before the microdialysis experiment animals were lightly anesthetized. A probe was carefully lowered through the guide cannula to the level of the dorsolateral striatum. The probe was secured in the guide cannula by epoxy. Artificial cerebral spinal fluid (aCSF) (140 mM NaCl, 3.36 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.45 mM NaH_2PO_4, and 4.85 mM Na_2HPO_4, pH 7.4) was perfused through the probes at 2 μl/min for 30 minutes and then the rate was lowered to 0.5 μl/min overnight. The animals had access to food and water throughout the night.

The next morning, the aCSF flow rate was increased to 2 μl/min for 30 minutes prior to the collection of baseline samples and the food and water were removed. Four, 15-minute baselines samples were collected in the morning. In the first experiment, aCSF containing high magnesium, no calcium, and the calcium chelator EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid) (140 mM NaCl, 3.36 mM KCl, 2.5 mM $MgCl_2$, 1.45 mM NaH_2PO_4, 4.85 mM Na_2HPO_4, and 2.0 mM EGTA, pH 7.4) was then perfused through the probe and eight additional 15-minute samples were collected. This altered aCSF was used to determine if the lack of calcium would reduce the amount of glutamate in the extracellular space. In the second microdialysis experiment, a challenge injection of either vehicle, 15 mg/kg cocaine, or 30 mg/kg cocaine was administered during microdialysis and then eight, 15-minute samples were collected.

Figure 1A is a representative coronal section from the striatum of a rat used in the microdialysis experiment. The probes had to be located in the dorsolateral striatum in order for the data from that animal to be included in the data analysis. Figure 1B shows the probe placements for all animals used in the microdialysis experiments. The rats were placed in the microdialysis

chambers overnight before sample collection. Sample collection did not disturb the rats in their chambers. In addition, switching the aCSF solutions and injecting the rats did not produce a fluctuation in extracellular glutamate. The baseline values of extracellular glutamate were very stable and this is evidenced by the low standard errors reported in the first two tables.

HPLC Detection of Glutamate

The dialysate within each vial collected was analyzed for glutamate concentrations. Glutamate concentration was determined using a Hewlett Packard HPLC 1090 and a Hewlett Packard 1046A Programmable Fluorescence Detector. Dialysates were derivatized with o-phthalaldehyde (OPA) and chromatographed as previously reported (Meshul et al., 1999;Meshul et al., 2002;Schuster, 1988). The sensitivity for glutamate was in the subpicomole range.

Probe Placement Verification and Data Analysis

After the completion of the microdialysis experiment, animals were transcardially perfused with glutaraldehyde fixative (see Electron Microscopy section). Sections (100 μm) of the left striatum were cut using a vibratome and stained with thionin to histologically verify the probe placement within the dorsolateral striatum. If the probe placement was not in the dorsolateral quadrant of the striatum, then the animal was excluded from the study. The concentration of dialysate within each sample was separately averaged at each time point and then a grand mean determined for each group. The values are expressed as the mean ± SEM in picomoles/ μl.

Statistical Analysis

In the first experiment, the levels of extracellular glutamate for each group of cocaine-treated rats (1, 2, 3, and 14 day groups) were compared to their respective control group.

Statistical significance between the each cocaine-treated group and its respective control group was determined using the Student's *t*-test. The Student's *t*-test was also used to compare the extracellular glutamate values between the altered aCSF treatment and the baseline values for each group. In the second experiment, the Student's *t*-test was used to compare the extracellular glutamate levels of the vehicle or cocaine-injected groups to their respective baseline values. For quantitative electron microscopy, outliers more than two standard deviations from the mean were eliminated. Approximately 1 outlier was eliminated out of every 40 density measurements (2.5%). The mean density of glutamate immunogold labeling was compared between the cocaine-treated group and its respective control group using a Student's *t*-test. For the acute experiment (Table 4), the density of glutamate immunolabeling for each group was determined using a one-way ANOVA because all three groups were treated at the same time. Statistical significance was set at $p < .05$. JMP (version 5.0.1.2) software was used to perform all statistical analysis.

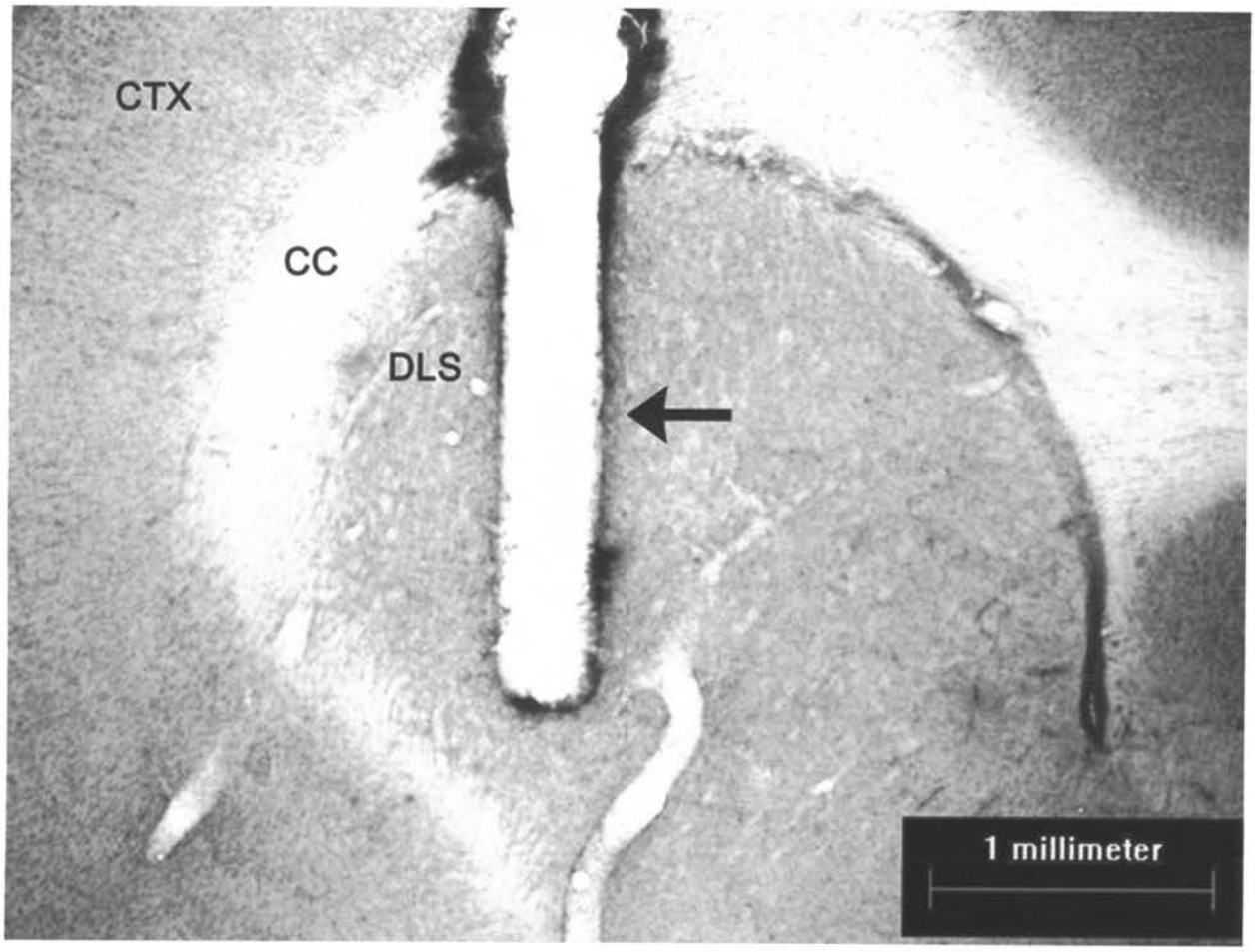


Figure 1A: A representative photomicrograph of a microdialysis probe placement in the dorsolateral striatum of the rat. The arrow points to the cellulose probe tip where the extracellular fluid is collected. The left dorsolateral striatum was cut into 100 μm sections and stained with thionin. Abbreviations are CTX for cortex, CC for corpus callosum, and DLS for dorsolateral striatum. Scale bar is 1 mm. (Note: dorsal is up, lateral is to the left).

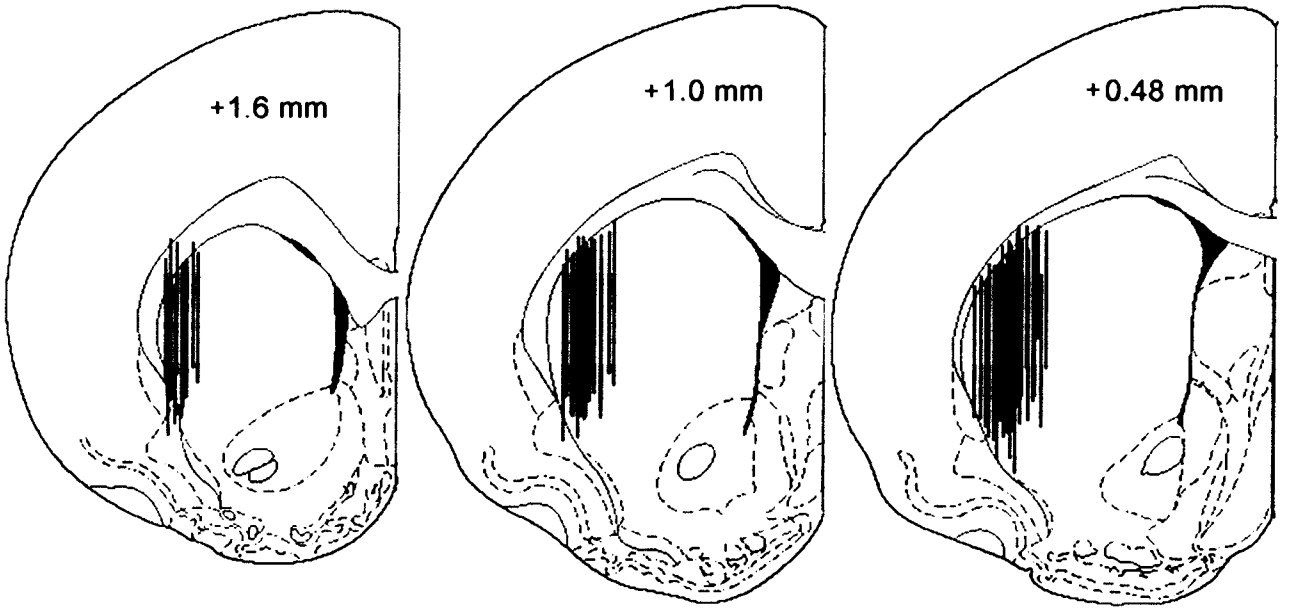


Figure 1B: Representations of the probe placements for all of the rats used in the microdialysis experiments. The probes were located in the dorsolateral striatum and ranged from 1.6 mm to 0.48 mm anterior to bregma (Paxinos and Watson, 2005).

Results

In Vivo Microdialysis

The levels of extracellular glutamate in the dorsolateral striatum were measured after a single i.p. injection of 15 mg/kg cocaine (Table 1). Figure 2 shows the percent change in baseline extracellular glutamate after a single cocaine injection. One day after acute cocaine administration, extracellular glutamate increased 61% compared to the group injected with vehicle 1 day previously $t(6) = 3.88$, $p = .0082$ (n = 8 for the cocaine-treated group, n = 7 for the control group). There was no significant difference between extracellular glutamate in rats injected with cocaine or vehicle 2 days prior to the microdialysis experiment $t(6) = 0.96$, $p = .37$ (n = 8 for the cocaine-treated group, n = 7 for the control group). Extracellular glutamate decreased in rats injected with 15 mg/kg cocaine 3 days prior to the microdialysis session compared to the vehicle-treated group $t(6) = 5.27$, $p = .0019$ (n = 16 for the cocaine-treated group, n = 7 for the control group). Two weeks after a single cocaine injection, extracellular glutamate decreased 29% compared to the vehicle-treated group $t(6) = 3.78$, $p = .0092$ (n = 7 for the cocaine-treated group, n = 8 for the control group). There were no noticeable differences in behavior between any of the groups on the day of the microdialysis experiment.

At each time point, there was a separate group of rats for the cocaine and vehicle-treated groups. The microdialysis samples from the vehicle-treated and cocaine-treated groups were analyzed together at each time point. The average control values of extracellular glutamate were 2.84 pmol/μl for the 1 day control group, 0.83 pmol/μl for the 2 day control group, 1.08 pmol/μl for the 3 day control group, and 2.59 pmol/μl for the 14 day control group. Baseline glutamate values are known to differ between groups of control animals due to biological variability. Therefore, the table shows the changes between groups as a percent of control.

Table 1. Changes in Extracellular Glutamate after a Single Cocaine Injection and Altered aCSF Exposure[a,b]

Group	% of Control of Extracellular Glutamate[a]	% Reduction in Glutamate with Altered aCSF[b]
1 Day Following Cocaine	163.66 ± 9.4*	28.2**
2 Days Following Cocaine	112.87 ± 10.2	13.7
3 Days Following Cocaine	65.22 ± 3.1*	35.1**
14 Days Following Cocaine	72.09 ± 7.1*	N.D.

[a]Values are a percent of control of extracellular glutamate ± S.E.M. *$p < .05$ compared to each control group's baseline levels. [b]Values are a percent reduction in extracellular glutamate ± S.E.M. after perfusing with the altered (high magnesium, no calcium) aCSF. **$p < .05$ compared to each group's baseline levels. N.D. signifies that the values were not determined for this group.

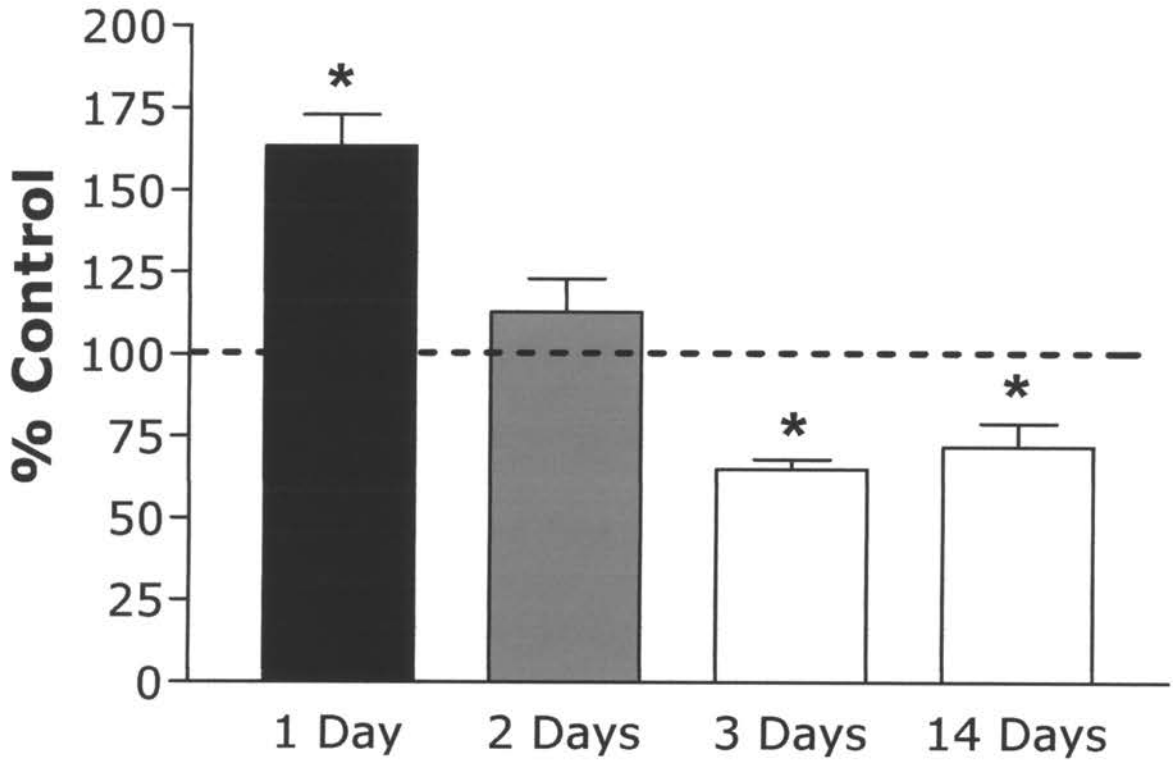


Figure 2: Extracellular glutamate in the dorsolateral striatum increased 1 day after a single cocaine injection of cocaine (15 mg/kg, i.p.), and then decreased 3 to 14 days after an acute cocaine exposure (n = 7-12 per group, * = $p < 0.05$). Each bar represents the percent of control value (dotted line) resulting from a single cocaine treatment (% control ± S.E.M.).

To determine the influence of calcium on the extracellular levels of glutamate, altered artificial cerebral spinal fluid (aCSF) containing no calcium, high magnesium, and a calcium chelator (EGTA) was perfused through the microdialysis probe after collecting baseline samples in normal aCSF. Table 1 shows the percent change in extracellular glutamate after the perfusion of the altered (high magnesium, no calcium) aCSF. The altered aCSF was not used in the group that had been administered acute cocaine 14 days previously. There was a significant decrease of 28.2% after perfusion of the altered aCSF in the group that had received a cocaine injection 1 day previously $t(6) = 2.46$, $p = .05$ (n = 5). Rats that had received cocaine 2 days previously had a 13.7% decrease in their extracellular glutamate levels in the altered aCSF $t(6) = 1.46$, $p = .19$ (n = 8). A 35.1% decrease in extracellular glutamate was found in rats that had received a cocaine injection 3 days previously $t(6) = 6.13$, $p = .0009$ (n = 7). Although the altered aCSF produced a modest decrease in the cocaine-treated groups, only a 5.1% reduction was seen in all of the control animals $t(6) = 0.68$, $p = .52$ (n = 7).

To determine if the cocaine-induced increase in extracellular glutamate develops immediately after the injection, drug-naïve rats were injected with vehicle, 15 mg/kg cocaine, and 30 mg/kg cocaine after collecting baseline samples. Within the two-hour sampling period after a single injection of cocaine or vehicle, there was no significant difference in the groups injected with vehicle $t(10) = 1.17$, $p = .27$ (n = 5), 15 mg/kg cocaine $t(10) = 0.48$, $p = .64$ (n = 6), and 30 mg/kg cocaine $t(10) = 0.25$, $p = .81$ (n = 5) as compared to their respective baseline values (Table 2). This suggests that the cocaine-induced changes described above require approximately 24 hours to develop. The average baseline value of extracellular glutamate was 0.56 pmol/μl for the vehicle-treated group, 0.33 pmol/μl for the 15 mg/kg cocaine-treated group, and 0.50 pmol/μl for the 30 mg/kg cocaine-treated group.

Table 2. Extracellular Glutamate After Acute Cocaine Exposure[a]

Group	% of Baseline from Challenge Injection of Cocaine
Vehicle	114.82 %
15 mg/kg Cocaine	90.37 %
30 mg/kg Cocaine	101.82 %

[a]Values are a percent change from baseline extracellular glutamate due to the challenge cocaine injection.

Nerve Terminal Glutamate Immunolabeling

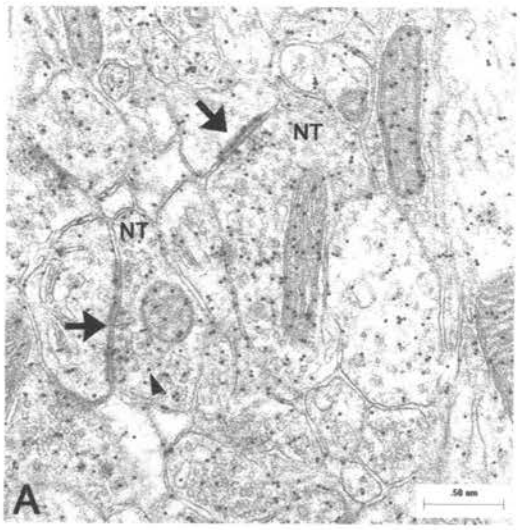
The density of glutamate immunogold labeling in striatal nerve terminals associated with the synaptic vesicle pool was measured using immunogold electron microscopy. Glutamatergic nerve terminals are identified by the presence of gold particles that are associated with vesicles and by the formation of an asymmetrical contact onto a dendritic spine (Figure 3). The asymmetrical contacts are denoted by the presence of a prominent postsynaptic density. Nerve terminals making an asymmetrical synaptic contact have been shown to be excitatory and glutamatergic in the striatum (Meshul et al., 1999).

The rats from the first microdialysis experiment were transcardially perfused and the rostral aspect of the right dorsolateral caudate nucleus from each animal was used for immunogold electron microscopy. Table 3 shows the density of striatal nerve terminal glutamate immunolabeling expressed as a percent of the control values. There was no significant difference between the groups treated with 15 mg/kg cocaine 1, 2, 3, or 14 days previously and their respective control groups (n = 5-7 for each group). The density of immunogold labeling was not affected by nerve terminal size because there were no differences in the areas of the nerve terminals between any of the groups (data not shown).

Pronounced changes in striatal Fos-like immunoreactivity 2 hours after an acute cocaine injection have been reported (Graybiel, 1990). To determine if immediate changes in nerve terminal glutamate labeling could be found after an acute cocaine injection, rats were injected with vehicle, 15 or 30 mg/kg of cocaine (n = 4 for each group). Two hours after the injection, the rats were transcardially perfused and the tissue was processed for immunogold electron microscopy. Table 4 suggests a trend towards a non-significant decrease in the density of immunogold labeling between the control group and the cocaine-treated groups $F(2, 9) = 3.37$, $p = .08$.

To determine if repeated cocaine administration produces changes in nerve terminal glutamate immunolabeling similar to what we have reported in the nucleus accumbens

Figure 3: Representative electron micrographs of the dorsolateral striatum of the rat. An identified nerve terminal (NT) forms an asymmetrical synaptic contact onto a dendritic spine as noted by the prominent postsynaptic density (black arrow). The gold particles appear as black dots on the micrographs, which are associated with synaptic vesicles (black arrowhead). (A) Micrograph of the neuropil of a control animal. Micrographs of the neuropil from a rat that had been injected with 15 mg/kg cocaine 1 day previously (B), 2 days previously (C), 3 days previously (D), 14 days previously (E). Note the lack of differences in the density of nerve terminal glutamate immunolabeling between the groups. Scale bar is 0.5 μm.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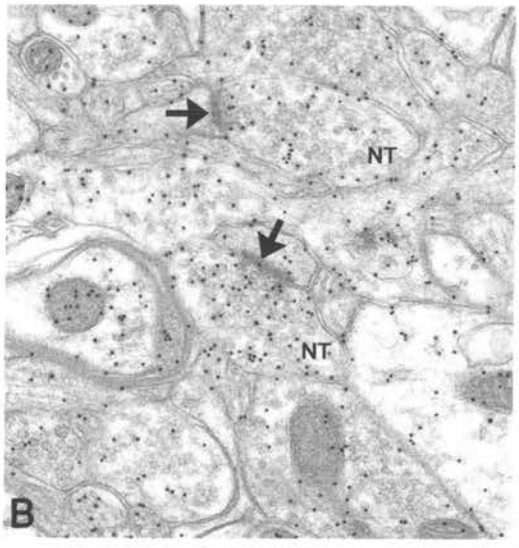
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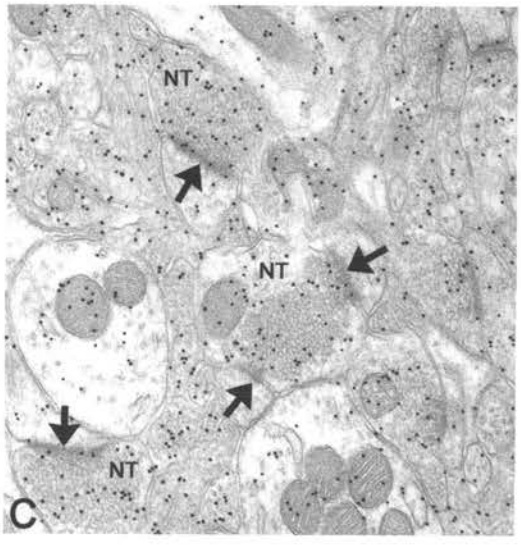
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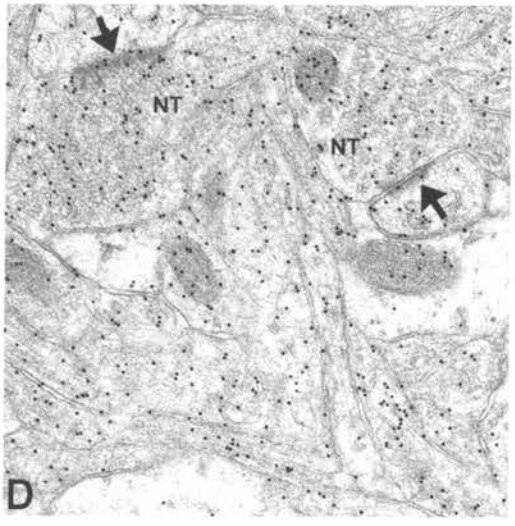
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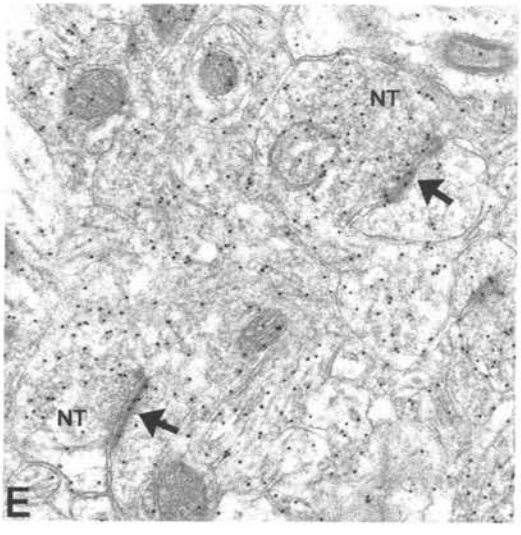
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Table 3. Striatal Nerve Terminal Glutamate Immunolabeling after a Single Cocaine Exposure[a]

Group	% of Control of Glutamate Immunogold Labeling
1 Day Following Cocaine	106.00 ± 8.6
2 Days Following Cocaine	107.53 ± 8.6
3 Days Following Cocaine	124.63 ± 7.1
14 Days Following Cocaine	76.66 ± 11.3

[a]Values are a percent of control of immunogold labeling ± S.E.M.

Table 4. Striatal Nerve Terminal Glutamate Immunolabeling after Acute and Intermittent Cocaine Exposure[a]

Group	Density of Glutamate Immunogold Labeling After Acute Treatment	Density of Glutamate Immunogold Labeling After Intermittent Treatment
Vehicle	74.89 ±4.7	53.30 ± 4.29
15 mg/kg Cocaine	65.38 ±7.2	48.26 ± 3.69
30 mg/kg Cocaine	55.30 ±2.3	N.D.

[a]Values are the mean number of gold particles/μm^2 terminal area ± S.E.M. N.D. signifies that the values were not determined for this group. There were no significant differences between any of the groups.

(Kozell and Meshul, 2001), rats were injected with vehicle (n = 5) or 15 mg/kg cocaine (n = 8) for 7 days and then withdrawn for 14 days. Table 4 shows that there was no significant difference in the density of nerve terminal immunogold labeling between the vehicle-treated and cocaine-treated groups $F(1,11) = 0.85$, $p = 0.37$.

Discussion

The most unique finding of this study is that a single injection of cocaine produced long-lasting, time-dependent changes in extracellular glutamate in the dorsolateral striatum as measured by *in vivo* microdialysis. Extracellular glutamate initially increased 1 day after a single cocaine injection and then 3 days later, extracellular glutamate decreased for at least 2 weeks. These changes in extracellular glutamate cannot be explained by changes in nerve terminal glutamate immunolabeling, nor does nerve terminal glutamate immunolabeling change in response to withdrawal from intermittent cocaine treatment.

Effects of acute cocaine on extracellular glutamate

Several laboratories have measured the immediate changes in extracellular glutamate after acute cocaine in the striatum using microdialysis (Reid et al., 1997;Pierce et al., 1996;Smith et al., 1995). Although extracellular glutamate was sampled from different striatal areas, no changes in extracellular glutamate were reported. These results agree with our findings and show that acute cocaine does not have an immediate effect on striatal glutamate as measured with microdialysis.

Long-lasting effects of a single cocaine exposure are not limited to glutamate. Similar results have been found for dopamine in the dorsal striatum. Peris and Zahniser (1987) obtained dorsal striatal slices from rats that had been injected with vehicle or 10 mg/kg cocaine 1 day to 14 days previously. They reported an augmentation of amphetamine-stimulated release of [^{3}H]-dopamine in the cocaine-treated group as compared to the control group. This

effect was present 24 hours after a single cocaine exposure and persisted for 2 weeks post-injection. This study in combination with the present data suggests that acute cocaine has profound and long-lasting changes on two important striatal neurotransmitters, glutamate and dopamine.

The cocaine-induced increase in extracellular dopamine could alter the output of the striatum through modulating extracellular glutamate. Dopamine could preferentially bind to dopamine D-1 receptors located on the medium spiny neurons. Activated medium spiny neurons would increase the GABAergic inhibition to the substantia nigra pars reticulata and entopeduncular nucleus, thereby reducing the GABAergic inhibition onto the motor thalamus. This would result in an increase in the thalamocortical input to the dorsolateral striatum and effectively increase extracellular glutamate. The long lasting increase in striatal dopamine reported by Peris and Zahniser (1987) could therefore explain this trans-synaptic mechanism by which extracellular glutamate is increased 1 day after a single cocaine injection. The calcium-free aCSF reduced extracellular glutamate in the cocaine-treated animals (Table 1), which suggests the possibility that the cocaine-induced changes in extracellular glutamate could be mediated through the basal ganglia circuitry. Furthermore, the cocaine-induced increase in extracellular glutamate is blocked by a prior lesion of the motor thalamus, which would reduce the thalamocortical input to the dorsolateral striatum (McKee and Meshul, unpublished findings). These combined data suggest that the increase in extracellular glutamate 1 day after cocaine administration is mediated through a poly-synaptic mechanism in the basal ganglia circuitry.

Activation of the basal ganglia circuitry is not the likely mechanism by which extracellular glutamate decreases 3 to 14 days after a single cocaine injection. It is possible that the long term increase in extracellular dopamine after a single cocaine exposure could bind to dopamine D-2 receptors on corticostriatal terminals, effectively decreasing extracellular glutamate in the dorsolateral striatum. Glial cells also regulate extracellular levels of glutamate. Acute cocaine could cause astrocytes to proliferate, which could contribute to the changes in extracellular

glutamate. Glial fibrillary acidic protein (GFAP) is a structural marker that has been used to detect changes in mature astrocyte proliferation (Buckman and Meshul, 1999;Bowers and Kalivas, 2003). Withdrawal from repeated cocaine administration does not alter GFAP-positive cell counts in the dorsal striatum (Bowers and Kalivas, 2003), however the long-term changes in GFAP labeling in the dorsolateral striatum after acute cocaine exposure are not known. Astrocyte proliferation could increase the expression of glutamate transporters located on postsynaptic spines and glial cells. Glutamate/ aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) are located on glial cells and the excitatory amino acid carrier 1 (EAAC1) transporter is neuronal. An increase in glutamate transporters would enhance the removal of glutamate from the synaptic cleft, thereby decreasing extracellular glutamate. It is possible that glutamate transporter expression in the dorsolateral striatum is increased 3 to 14 days after a single injection of cocaine.

It has been reported that blocking NMDA receptors produces stereotypic motor activity (Takahata and Moghaddam, 2003;Schmidt, 1986;Danysz et al., 1994). Although glutamate receptor antagonist-induced stereotypies differ from psychostimulant-induced stereotypic behaviors, the decreased extracellular glutamate observed 3 days after a single cocaine exposure would reduce the glutamate available to NMDA receptors. It is possible that this decreased glutamatergic tone in the striatum could be responsible for sensitizing an animal to stereotypic behavior upon repeated exposure to cocaine. Overall, attenuated glutamatergic activity would likely decrease the striatal output via the direct pathway, thereby reducing voluntary movement.

Source of extracellular glutamate

The source of extracellular glutamate is controversial. Both the calcium-free aCSF and the glutamate immunolabeling experiment partially addressed the source of extracellular glutamate. The electron microscopy technique enables quantification of the relative density of immunolabeling in the vesicular glutamate pool. Cocaine did not significantly alter nerve

terminal glutamate immunolabeling within the synaptic vesicle pool. This suggests that either cocaine does not affect vesicular glutamate in dorsolateral striatal nerve terminals or cocaine induces a compensatory mechanism such as altered glutamate synthesis or reuptake that enables vesicular glutamate immunolabeling to appear relatively unchanged from the control group. These results differ from our previous reports demonstrating a decrease in glutamate immunolabeling in the nucleus accumbens after acute cocaine treatment (Kozell and Meshul, 2001). However, the significant decrease in extracellular glutamate induced by the altered aCSF in the cocaine groups suggests that there is a small but important calcium-dependent component to the extracellular pool of glutamate. The altered aCSF had little impact on the control (vehicle-treated) animals and this has been shown previously (Kreuter et al., 2004;Timmerman and Westerink, 1997;Baker et al., 2002;Pierce et al., 1996;Smith et al., 1995;Reid et al., 1997). This suggests that in the naïve rat, extracellular glutamate levels are not calcium and impulse dependent.

These combined data suggest that the vesicular, calcium-dependent pool of glutamate is not the main source of extracellular glutamate. It has been reported that long-term changes in extracellular glutamate are calcium-independent and mediated through the cystine/ glutamate antiporter in the ventral striatum (Pierce and Kalivas, 1997;Baker et al., 2002). The antiporter is a sodium and calcium-independent transporter that exchanges extracellular cystine for intracellular glutamate (Baker et al., 2003). Inhibition of the cystine-glutamate antiporter reduces extracellular glutamate by 60%, suggesting that the majority of glutamate release is neuronal but calcium-independent (Baker et al., 2002).

Conclusions

The fact that acute cocaine alters both glutamate and dopamine demonstrates that these neurotransmitters are important for mediating cocaine-induced changes in the basal ganglia. Both glutamate receptor antagonists and dopamine receptor agonists produce psychostimulant-induced stereotypy (Canales and Graybiel, 2000;Danysz et al., 1994). Changes in

glutamatergic tone could enable stereotypic behavior (Takahata and Moghaddam, 2003) and drug-related habit formation (Hyman et al., 2001;Packard, 1999). In addition, it has been suggested that the initial response to cocaine is critical for determining whether an individual will be likely to administer cocaine again (Volkow et al., 1999;Davidson et al., 1993). Therefore, long-term changes in striatal glutamate induced by a single cocaine exposure could lead to a predisposition for repeated cocaine administration.

The mechanism for these biphasic changes in extracellular glutamate resulting from a single injection of cocaine is unknown. An increase in striatal glutamate could be caused by the activation of the glutamatergic input to the dorsolateral striatum. In fact, activation of the motor thalamus would increase the glutamatergic input to the sensorimotor cortex. The sensorimotor cortex provides a prominent glutamatergic input into the dorsolateral striatum. Activation of the thalamocortical pathway could increase extracellular glutamate in the dorsolateral striatum 1 day after cocaine treatment. Therefore, it is hypothesized that blockade of the motor thalamus would block the cocaine-induced increase in striatal glutamate 1 day after cocaine treatment. In addition, the increase in striatal glutamate could be associated with a decrease in the glutamate transporters that take up glutamate from the extracellular space. There could be a decrease in glutamate transporters available for glutamate reuptake, a decrease in the glial cells that the glutamate transporters are located on, or both. Therefore it is also hypothesized that the increase in striatal glutamate 1 day after cocaine treatment is associated with a decrease in astrocytes and glutamate transporters.

Chapter 2

Lesion of the motor thalamic nucleus blocks acute cocaine-induced changes in striatal glutamate

Abstract

Previous experiments have shown that a single injection of cocaine produces an increase in extracellular glutamate in the rat dorsolateral striatum 1 day after the acute cocaine was administered (McKee and Meshul, 2005). However, the neuronal and glial contributions to the increase in striatal glutamate are unknown. Activation of the thalamocortical input to the dorsolateral striatum could increase extracellular glutamate. It was hypothesized that the thalamo-cortico-striatal pathway may be involved in producing the acute cocaine-induced increase. We investigated this hypothesis by electrolytically lesioning the motor thalamus of rats prior to administration of an acute cocaine or vehicle injection. One day after the cocaine or vehicle injection, extracellular glutamate was measured in the dorsolateral striatum of the rat using *in vivo* microdialysis. A motor thalamic lesion blocked the cocaine-induced increase in striatal glutamate and reduced extracellular glutamate to the level of the vehicle-treated group. The increase in striatal glutamate could be associated with a decrease in the sodium-dependent glutamate transporters that take up glutamate from the extracellular space. In addition, the density of glial cells that express the glutamate transporters could also be decreased. It was hypothesized that the increase in striatal glutamate was associated with a decrease in glutamate transporters and glial cells. Glial fibrillary acidic protein (GFAP) and glutamate transporter immunolabeling were carried out on rats that had received either a vehicle or 15 mg/kg cocaine injection 1 day previously. We found that GFAP and glutamate/aspartate transporter (GLAST) immunolabeling was significantly decreased in the dorsolateral striatum of cocaine-injected rats as compared to the vehicle-treated group. Taken together, a single cocaine injection caused dynamic neuronal and glial changes.

Introduction

Cocaine is a powerful drug of abuse that has long-lasting effects on striatal glutamate after just one exposure (McKee and Meshul, 2005). Cocaine does not cause immediate changes in extracellular glutamate in the dorsolateral striatum (McKee and Meshul, 2005;Reid et al., 1997). However, 1 day after a single systemic cocaine injection, extracellular glutamate increases in the dorsolateral striatum (McKee and Meshul, 2005).

The dorsolateral striatum receives glutamatergic input from the sensorimotor cortex (McGeorge and Faull, 1989). The corticostriatal terminals form excitatory synaptic contacts onto the spines of medium spiny neurons in the dorsolateral striatum (Smith and Bolam, 1990). These medium spiny neurons are the main projection neurons for the striatum, and hence glutamate is an important modulator of striatal output. The dorsolateral striatum is the major output nucleus for the basal ganglia, which are involved in motor control and reward.

Striatal medium spiny neurons project via the indirect pathway to the globus pallidus externus (indirect pathway) and via the direct pathway to the substantia nigra pars reticulata (SNr) and entopeduncular nucleus, which is the homologue of the globus pallidus internus in the rat. The entopeduncular nucleus sends an inhibitory GABAergic projection to the ventromedial (VM) nucleus of the thalamus. The VM or motor thalamus sends a glutamatergic projection to the sensorimotor cortex, which affects the cortical input to the dorsolateral striatum (Meshul et al., 1996). Overall, increased glutamatergic activity would likely increase the output of the medium spiny neurons, thereby enhancing activity of the basal ganglia and the thalamocortical circuit.

Activation of the corticostriatal pathway leads to an increase in extracellular glutamate in the dorsolateral striatum (Meshul et al., 1996). Elevated extracellular glutamate increases the stimulation of the medium spiny neurons and thereby increases GABAergic inhibition of the entopeduncular nucleus and SNr via the direct pathway. This removes the inhibition to the

motor thalamus, and increases glutamate release in the sensorimotor cortex. Conversely, stimulation of the striatopallidal neurons via the indirect pathway increases GABAergic inhibition of the GPe. This reduces the inhibition of the subthalamic nucleus, which then activates the entopeduncular nucleus and the EPN. Hence, if the increase in striatal glutamate occurs through the basal ganglia circuitry, the direct pathway is activated, not the indirect pathway.

Less known are the effects of acute cocaine treatment on glial cells and the glutamate transporters located on them. Glial fibrillary acidic protein (GFAP) is a structural marker that has been used to detect changes in mature astrocyte proliferation (Buckman and Meshul, 1999;Bowers and Kalivas, 2003). Withdrawal from repeated cocaine administration does not alter GFAP-positive cell counts in the dorsal striatum (Bowers and Kalivas, 2003), however the changes in GFAP labeling after acute cocaine exposure are not known.

Three glutamate transporters have been identified in the rat (Rothstein et al., 1994). Glutamate/ aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) are located on glial cells and the excitatory amino acid carrier 1 (EAAC1) transporter is localized to the postsynaptic spine. Increased extracellular glutamate may be caused by a decrease in the density of striatal glutamate transporters. Fewer glutamate transporters lead to a reduction in the removal of glutamate from the extracellular space.

Previously we reported that a single cocaine injection increases extracellular glutamate in the rat dorsolateral striatum 1 day later (McKee and Meshul, 2005). Furthermore, it was shown that the increase in striatal glutamate was calcium-dependent, but did not result from changes in striatal nerve terminal glutamate immunolabeling. This suggests that extracellular glutamate in the cocaine-stimulated striatum is regulated partially by a neuronal, non-vesicular mechanism and that there is more than one mechanism by which cocaine induces an increase in striatal glutamate. In this report, we seek to determine if both neuronal and glial changes account for the acute cocaine-induced increase in striatal glutamate.

We hypothesized that the cocaine-induced increase in striatal extracellular glutamate is due to an increase in the activation of the thalamocortical projection to the striatum or to a decrease in striatal glutamate transporters. A lesion of the motor thalamus impairs the activation of the thalamocortical projection, subsequently decreasing the activity the corticostriatal pathway. To investigate the role of the motor thalamus in cocaine-induced increase in striatal glutamate, changes in extracellular glutamate were measured in motor thalamic and sham-lesioned animals after acute cocaine treatment. To investigate the role of astrocytes and glutamate transporters in the cocaine-induced increase in extracellular glutamate, the relative density of GFAP, GLT-1, EAAC1, and GLAST immunolabeling were determined 1 day after a single cocaine injection.

We found that an electrolytic lesion of the motor thalamus blocked the increase in striatal glutamate 1 day after a single cocaine injection. GFAP and the glial glutamate transporter GLAST immunolabeling was decreased 1 day after a single cocaine exposure.

Methods

Subjects

Male Sprague-Dawley rats (3 months old, Harlan, Indianapolis, IN) were housed at the Veterinary Medical Unit at the Veteran's Administration Medical Center in Portland, Oregon. Rats were housed in pairs in clear plastic cages with corncob bedding. Animals were maintained in a temperature and light controlled room on a 12-hour light/ dark cycle, with access to food and water ad libitum. All animals were drug and experimentally naïve at the beginning of the experiments. Care was taken to minimize the number of animals used and to reduce animal discomfort. The same animals were used for both the immunohistochemical and locomotor activity experiments to reduce the number of animals needed in the experiments. All procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory

Animals and were approved by the Veteran's Affairs Medical Center's Institutional Animal Care and Use Committee (IACUC).

Drugs

In all experiments, rats were injected with 15 mg/kg cocaine hydrochloride (Coc) (Sigma-Aldrich, St. Louis, MO) dissolved in sterile water or 0.2 ml sterile water for vehicle-treated (Veh) animals intraperitoneally.

Electrolytic Lesions

All animals subjected to electrolytic lesions simultaneously underwent guide cannula implantation to reduce the number of surgeries for each animal. Rats were anesthetized with 5% ketamine and 0.5% xylazine (1 ml/kg, i.p.) and their heads shaved and placed in a Stoelting stereotaxic apparatus. Their skulls were exposed by a small incision made on the skin covering their heads and two holes were drilled through the skull above the ventromedial (VM) nucleus of the thalamus for electrode placement. The VM nucleus of the thalamus was chosen because the substantia nigra projects to the VM and is considered the primary motor thalamus in the rat (Groenewegen et al., 1999). A small current was applied in two different locations to effectively lesion the rostral-caudal extent of the VM. The coordinates were respective to bregma: -2.3 and –2.7 mm rostral, +1.4 mm lateral, and –7.4 mm ventral on the left side of the brain and -2.1 and –2.5 mm rostral, -1.4 mm lateral, and –7.4 mm ventral on the right side of the brain (Paxinos and Watson, 2005). Optimal lesions were found on the left and right sides by varying the rostral-caudal coordinates slightly. A SNE-300 stainless steel monopolar electrode with a 0.1 mm tip was lowered into the VM nucleus (Rhodes Medical Instruments, Woodland Hills, CA). Upon proper insertion of the electrode, 0.5 mA current was applied for 2 seconds (UGO Basile). After the two sites were lesioned, the needle was removed and the guide cannulae implantation surgery was carried out (see below). Sham lesioned animals had the hole drilled into the skull but the needle was not inserted into the brain.

Cannula Implantation

Anesthetized animals had their heads shaved and were placed into the Stoelting stereotaxic apparatus. The skin above their skulls was cut and their skulls were exposed. A small hole was drilled using a dental drill at the following coordinates: (mm from Bregma) +0.5 rostral and +3.1 lateral for cannula placement above the dorsolateral striatum (Paxinos and Watson, 2005). Three additional holes were drilled and dental screws were placed in the holes. A stainless steel guide cannulae (15 mm long, 20-gauge) (Small Parts, Miami Lakes, FL) was lowered 0.5 mm from the surface of the skull without penetrating the dura. Dental cement was applied to the base of the guide cannulae and the three flathead screws to stabilize the guide cannula assembly. Epoxy was added on top of the dental cement to further stabilize the assembly. Then the skin around the assembly was stapled with clips to close the wound. The rats were allowed to recover for at least five days.

In Vivo Microdialysis

Dialysis probes were assembled according to Robinson and Wishaw (Robinson and Whishaw, 1988) with modifications (Meshul et al., 1999). The probes were 210 μm in diameter and the cellulose tips were 2 mm in length to effectively target the dorsolateral quadrant of the striatum. Probe recovery was tested by inserting the microdialysis probe into a solution of aCSF and 10 pmol/μl of glutamate. Three 10-minute samples were collected and analyzed via HPLC for the concentration of glutamate. The ability of the probes to recover in vitro glutamate ranged from 5 to 8%. An average of 6.2% of glutamate was recovered using the probes.

The day before the microdialysis experiment animals were lightly anesthetized with ketamine and xylazine as described above. A probe was carefully lowered through the guide cannula to the level of the dorsolateral striatum. The probe was secured in the guide cannula by epoxy. Artificial cerebral spinal fluid (aCSF) (140 mM NaCl, 3.36 mM KCl, 1.2 mM $CaCl_2$, 1.0

mM $MgCl_2$, 1.45 mM NaH_2PO_4, and 4.85 mM Na_2HPO_4, pH 7.4) was perfused through the probes at 2 μl/min for 30 minutes and then the rate was lowered to 0.5 μl/min overnight. The animals had access to food and water throughout the night.

The next moming, the food and water were removed from the dialysis chamber. The aCSF flow rate was increased to 2 μl/min for 30 minutes prior to the collection of dialysate samples. Four, 15-minute dialysate samples were collected.

HPLC Detection of Glutamate

The dialysate within each vial collected was analyzed for the concentration of glutamate. Glutamate concentration was determined using a Hewlett Packard HPLC 1090 and a Hewlett Packard 1046A Programmable Fluorescence Detector. Dialysates were derivatized with o-phthalaldehyde (OPA) and chromatographed as previously reported (Meshul et al., 1999;Meshul et al., 2002;Schuster, 1988). The sensitivity for glutamate was consistently in the subpicomole range. All of the samples were analyzed contiguously using the same standard curve. The concentration of dialysate within each sample was separately averaged at each time point and then a grand mean determined for each group. The values were expressed as the mean ± the standard error of the mean (SEM) in picomoles/ μl.

Probe Placement and Electrolytic Lesion Verification

After the completion of the microdialysis experiment, animals were transcardially perfused with glutaraldehyde fixative (see Electron Microscopy section). The rostral striata were cut into 100 μm thick sections using a vibratome and stained with thionin to histologically verify the probe placement within the dorsolateral striatum. If the probe placement was not in the dorsolateral quadrant of the striatum, then the animal was excluded from the study. The VM was cut into 70 μm sections and stained with thionin. The electrolytic lesions were bilateral, and the extent of the lesion was estimated in the following manner using the thionin-stained sections. For each section, the total area of the bilateral VM nuclei was determined using the

Imago-Pro software. A circle was drawn around the lesioned area using Image-Pro in each tissue section. Only the area where no cells were present was included in the lesion criteria. Gliotic cells were not included in the lesion criteria (see Figure 4A for example). Then the entire VM nucleus was outlined in each tissue section and Imago-Pro reported the areas for each of the outlined areas. The extent of the lesion was calculated for the left and right VM nuclei as the percent of the lesioned VM nucleus area divided by the total area of the VM nuclei. The extent of the lesion was determined as the average of the left and the right sides of the VM nuclei. The range was 25% to 92.9%, with a mean percentage of 60.7%.

Immunohistochemistry

Rats were transcardially perfused with acrolein fixative (1% acrolein in 2% paraformaldehyde diluted with 0.1M phosphate buffer) using a peristaltic pump (Cole Parmer, Vernon Hills). The dorsolateral striatum (from the rostral striatum to the anterior commissure) was sectioned (60 μm) with a vibratome (System 1000, Ted Pella, Redding, CA). Sections were incubated in 0.1% sodium borohydride in phosphate buffer to remove excess aldehyde groups and then washed in a blocking solution containing 0.1 M phosphate buffer, 10% normal goat serum, and 0.1% Triton X-100. Alternate tissue sections were incubated in the following primary antibodies overnight at 4°C. The guinea pig polyclonal primary antibody was GLAST (1:5000; Chemicon, Temecula, CA). The rabbit polyclonal primary antibodies were GLT-1 (1:30,000; Dr. David V. Pow, please see (Reye et al., 2002)) and EAAC1 (1:2500; Chemicon, Temecula, CA). GFAP (1:1000) was a mouse monoclonal antibody (Sigma, St. Louis, MO). Control slices were incubated in blocking serum without the primary antibody. Tissue was rinsed in Triton-X-free blocking solution and then incubated in the secondary antibody (biotinylated goat anti-rabbit, anti-mouse, or anti-guinea pig IgG diluted to 0.44% in 0.1 M phosphate buffer, 1:200) for 1.5 hours. Next the tissue was exposed to an avidin-biotin complex (Vector ABC kit) and enhanced with diaminobenzidine (DAB, Vector Peroxidase Substrate kit). The tissue was exposed to the nickel-enhanced DAB solution for 2 minutes for the GFAP and

GLT-1 antibodies, 3 minutes for the EAAC1 antibody, and 2.5 minutes for the GLAST antibodies. After rinsing the tissue in 0.1 M phosphate buffer, the tissue was mounted on gelatin-coated slides. The slides were allowed to dry overnight, and then cover-slipped with Protex.

Optical Density Measurements

Images were captured directly using a Zeiss Axioplan light microscope (Carl Zeiss Inc., Germany) with a 5x objective (final magnification was 62.5x). Relative optical density measurements were carried out using Image-Pro Plus software. The dorsolateral striatum was outlined using the Image-Pro software, and then the relative optical density value was obtained for this area. The mean density of at least 4 sections was determined for each antibody.

Statistical Analysis

In the microdialysis experiment, the levels of extracellular glutamate were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare differences between groups. For the immunohistochemical study, the optical density value for cocaine-treated groups were compared to their respective vehicle-treated group for each antibody and significance was determined using a using a Student's *t*-test. Statistical significance was set at $p < .05$. JMP (version 5.0.1.2) and Prism 4 (version 4.03) software was used to perform all statistical analysis.

Results

Motor Thalamus Lesions

An example of the bilateral lesion of the VM nucleus of the thalamus is demonstrated in the thionin-stained photomicrograph in Figure 4A. Figure 4B shows the extent of the electrolytic

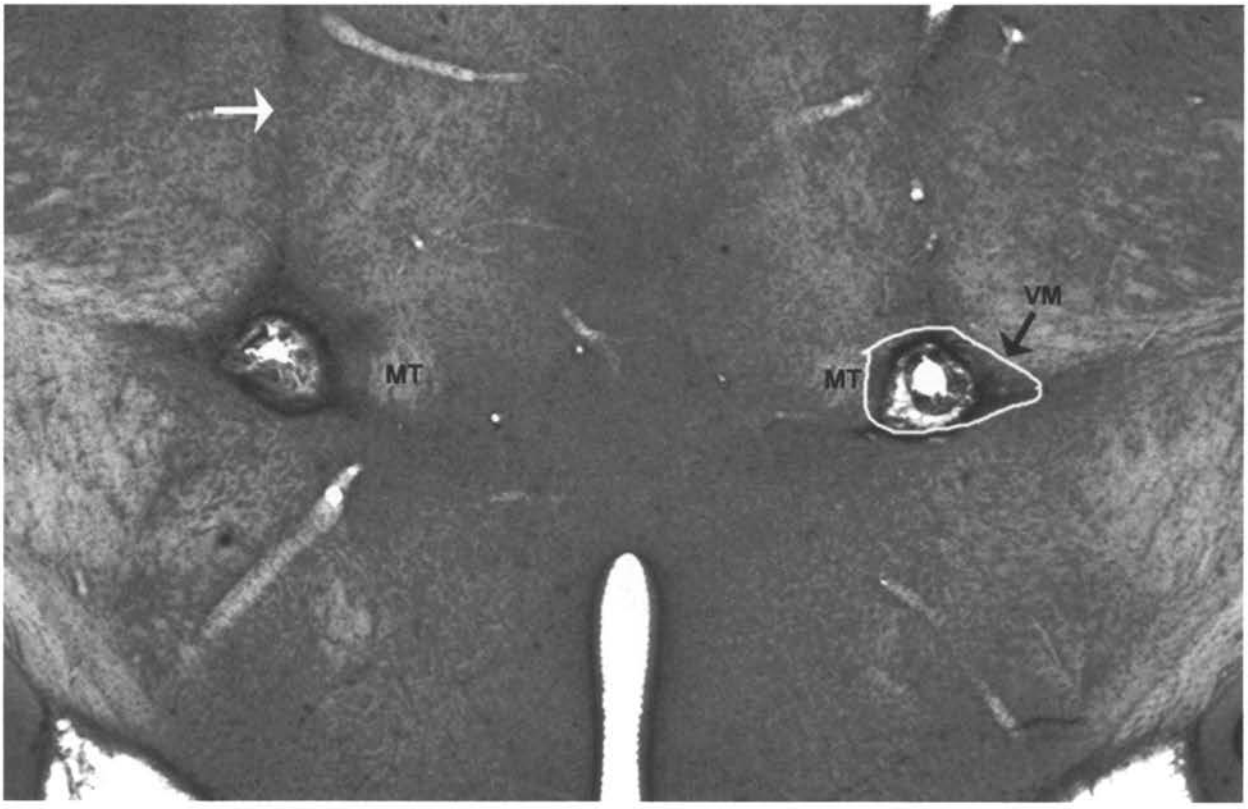


Figure 4A: A representative photomicrograph of a thionin-stained cross section shows the bilateral lesion of the ventromedial (VM) nucleus of the thalamus. The white arrow points to the needle tract and a white circle is drawn around the right VM nucleus. The mammillothalamic tract (mt) is labeled for orientation.

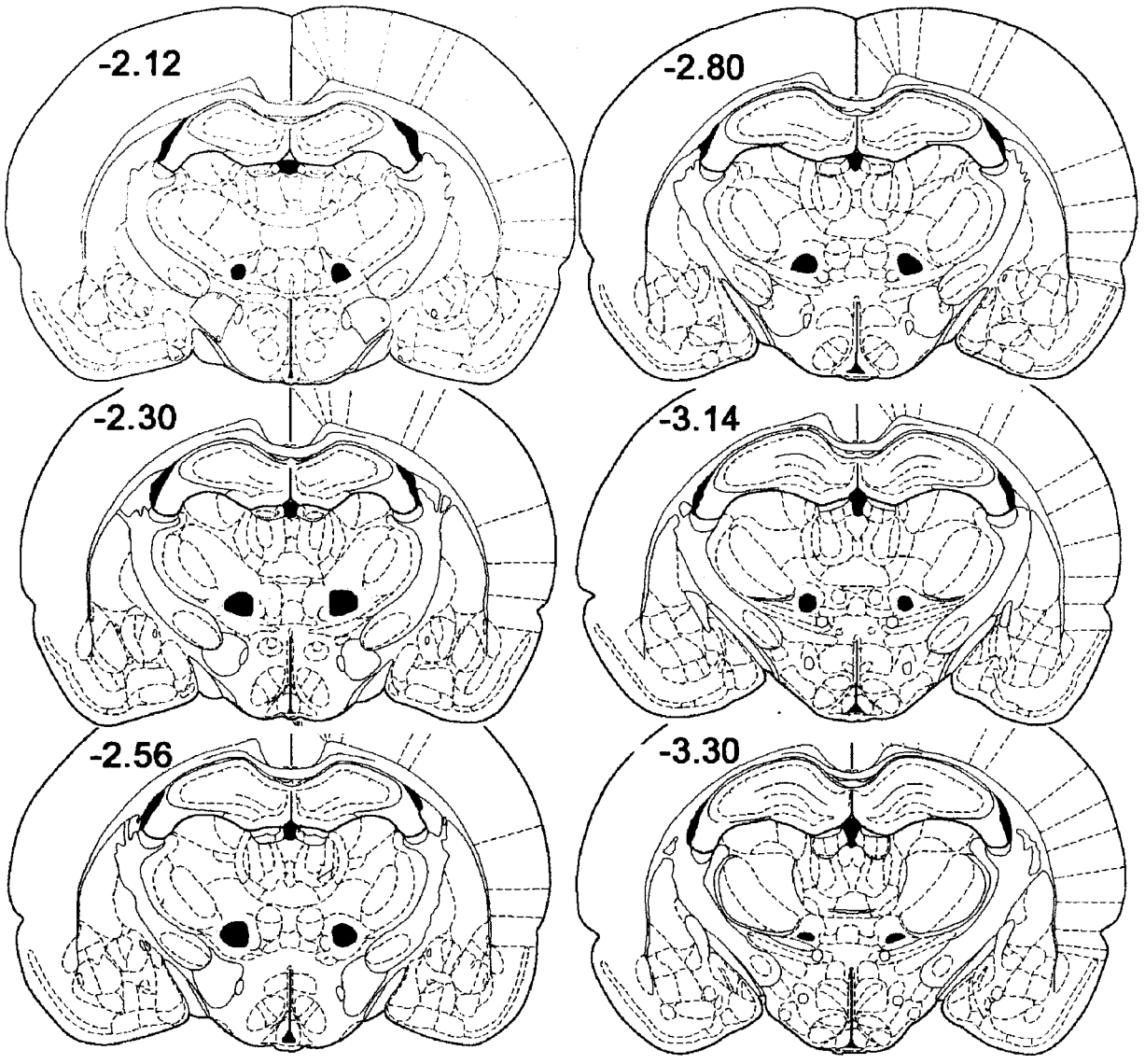


Figure 4B: Schematic representations illustrate the extent of the electrolytically lesioned VM nucleus of the thalamus. The black area shows the loss of cells and the gray area shows gliotic changes. The drawings were obtained from Paxinos and Watson, 2005 and the coordinates were the mm posterior from bregma. The most rostral drawing is in the top left corner and the most caudal section is in the bottom right corner.

lesion for all animals. The electrical current produced a significant lesion of the VM nucleus (61%). In addition, the area surrounding the site of the lesion showed gliotic changes. Neurons in the most posterior aspect of the ventromedial nucleus were usually spared. There were no obvious behavioral changes between VM-lesioned and sham-lesioned rats.

In Vivo Microdialysis

The probe placements for the rats used in the microdialysis experiment are illustrated in Figure 5. Figure 6 shows the effects of a motor thalamic lesion on extracellular glutamate in the rat dorsolateral striatum 1 day after acute cocaine administration. A lesion of the VM nucleus of the thalamus significantly reduced extracellular glutamate by 39.4% in the dorsolateral striatum in the cocaine naïve animals $F(3,12) = 81.84$, $p < 0.0001$ (Sham/Veh vs. VM/Veh). Extracellular glutamate was significantly increased by 15.6% 1 day after a single injection of cocaine in the sham-lesioned rats as previously reported (Sham/Coc vs. Sham/Veh) (McKee and Meshul, 2005). However, striatal glutamate was significantly decreased in VM-lesioned rats after 1 day after a single cocaine exposure as compared to sham-lesioned rats by 42.3% (VM/Coc vs. Sham/Coc). A lesion of the motor thalamus blocked the cocaine-induced increase in striatal glutamate.

Immunohistochemistry

The effects of a single cocaine injection on GFAP and glutamate transporter immunolabeling in the dorsolateral striatum were determined 1 day after the acute cocaine exposure (Figure 7). Figure 8 shows high magnification photographs of striatal immunolabeling for GFAP, GLAST, GLT-1, and EAAC1 in vehicle-treated and cocaine-treated rats. One day after an acute i.p. injection of 15 mg/kg cocaine, GFAP immunolabeling significantly decreased in the dorsolateral striatum as compared to vehicle-treated rats $t(68) = 2.75$, $p < 0.008$. GLAST immunolabeling was also significantly decreased in cocaine-treated rats as compared to

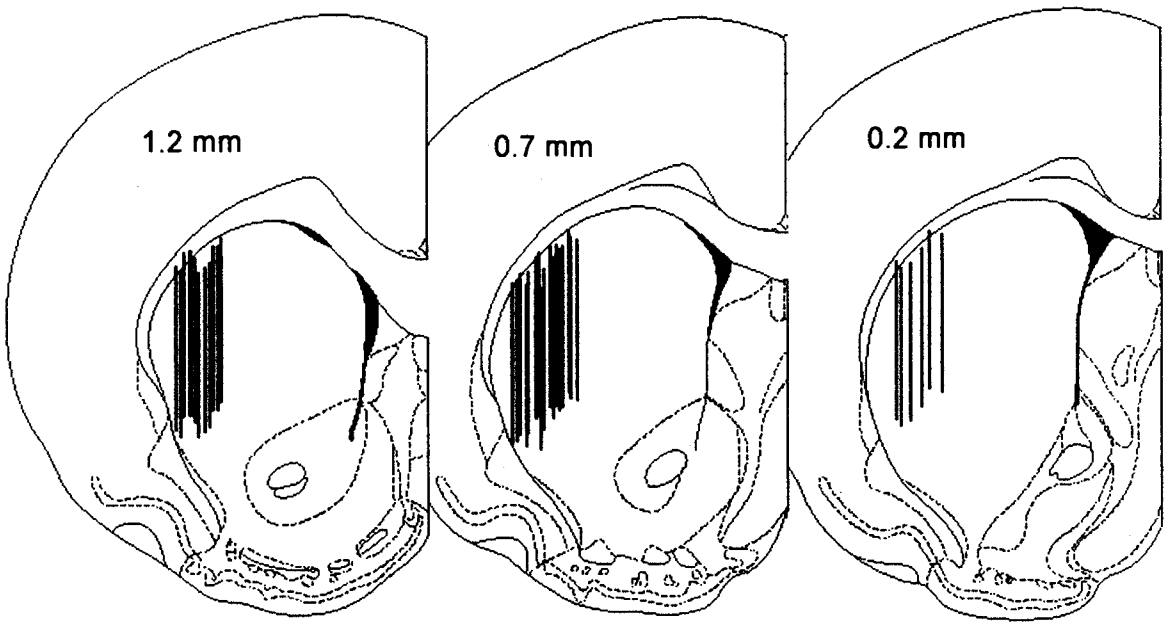


Figure 5: A schematic representation shows the locations of the microdialysis probe placements for all rats. The probes were located in the dorsolateral striatum and ranged from 1.2 mm to 0.2 mm anterior to bregma (Paxinos and Watson, 2005).

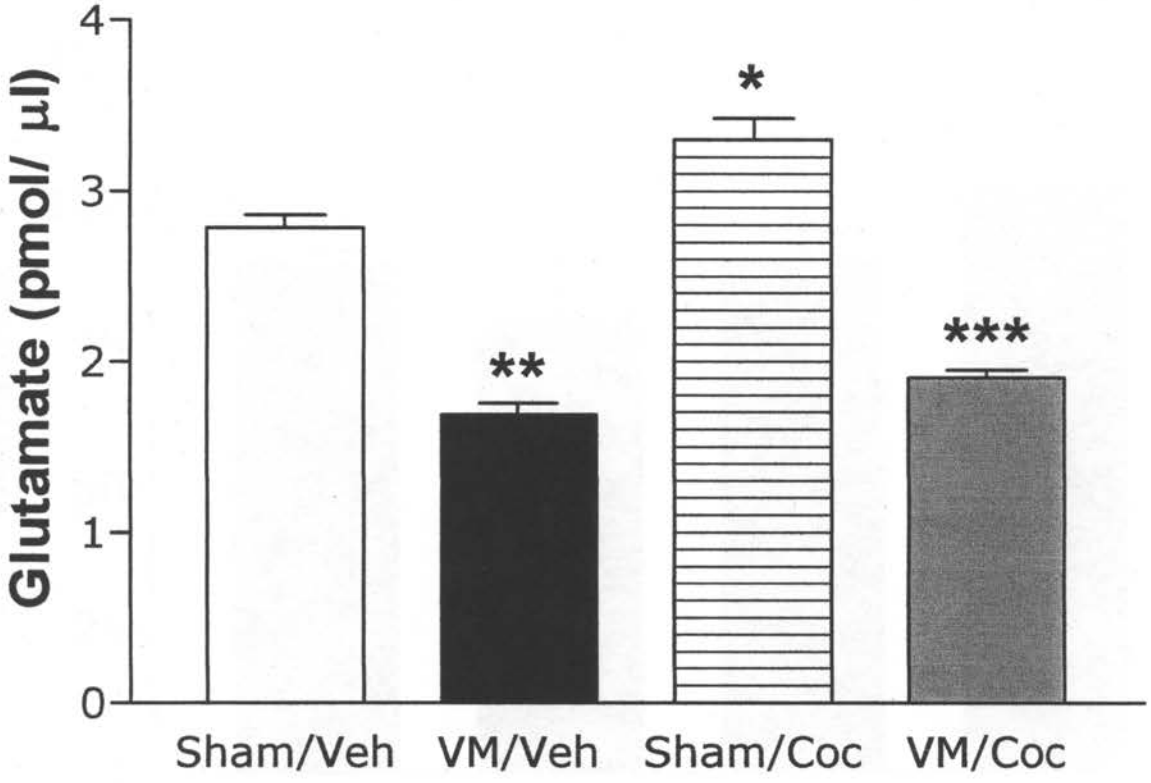


Figure 6: Electrolytic lesion of the motor thalamus blocked the increase in extracellular glutamate 1 day after a single i.p. injection of 15 mg/kg cocaine (n = 8-11 for each group). The motor thalamic lesion reduced extracellular glutamate in vehicle-treated rats (VM/Veh vs. Sham/Veh, ** = $p < 0.05$). A single injection of cocaine 1 day prior to microdialysis significantly increased extracellular glutamate in sham-lesioned rats (Sham/Coc vs. Sham/Veh, * = $p < 0.05$). However, the motor thalamic lesion prevented the cocaine-induced increase in extracellular glutamate (VM/Coc vs. Sham/Coc, *** = $p < 0.05$). Each bar represents extracellular glutamate in pmol/µl ± S.E.M.

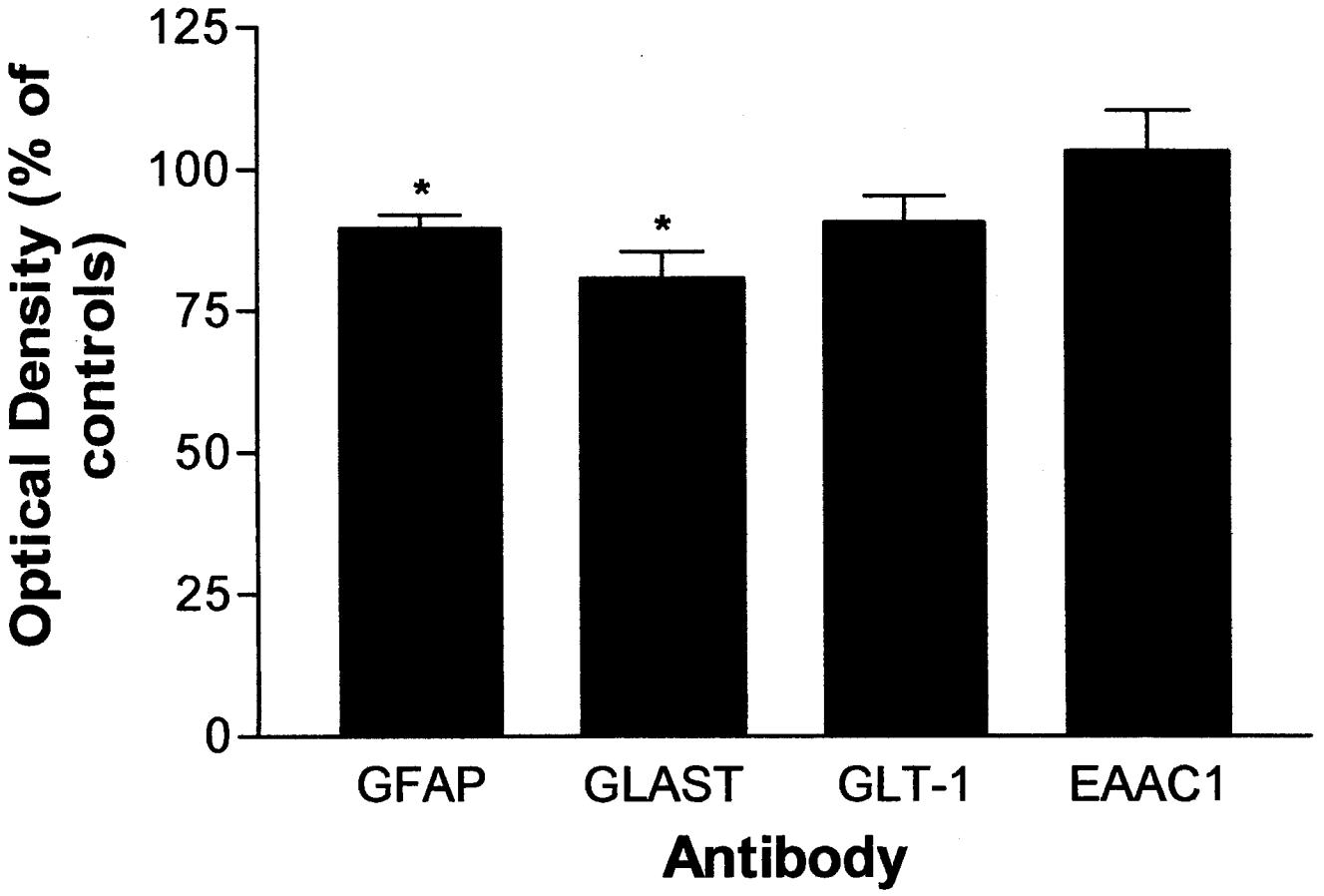
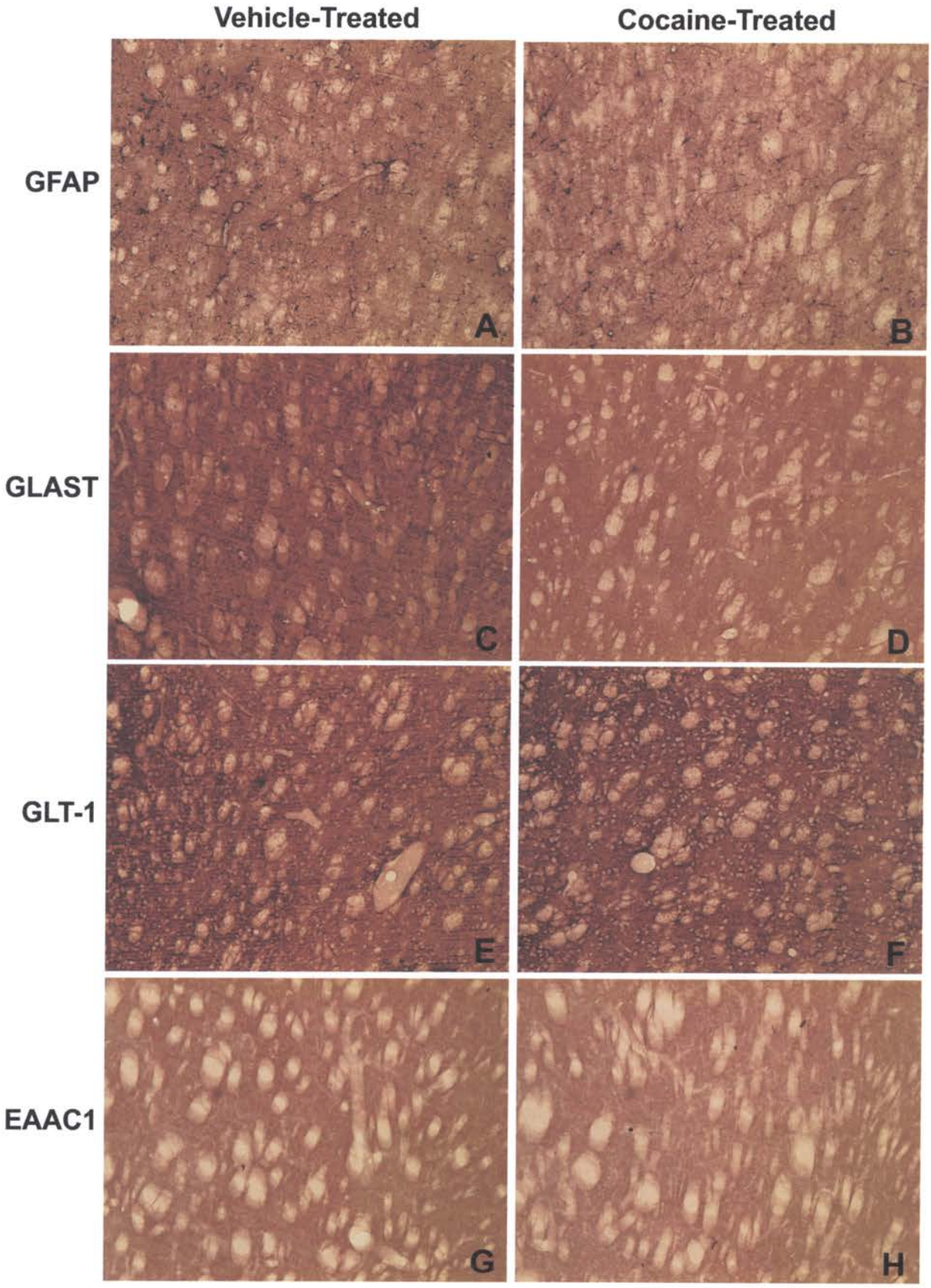


Figure 7: The relative density of GFAP and glutamate transporter immunolabeling in the dorsolateral striatum was decreased 1 day after a single cocaine exposure (n = 6 for each group). Each column represents the percent of control optical density value resulting from acute cocaine treatment (% control ± S.E.M.). Significant differences in the optical density of the cocaine-treated group as compared to its respective control group for each antibody is denoted by the asterick (* $p < 0.05$).

Figure 8: Representative photomicrographs of the GFAP and glutamate transporter immunolabeling of the dorsolateral striatum of the rat 1 day after a single cocaine or vehicle treatment. A, C, E, G are photographs demonstrating the immunolabeling of GFAP, GLAST, GLT-1 and EAAC1 in the DLS 1 day after a vehicle injection, respectively. B, D, F, H are photographs demonstrating the immunolabeling of GFAP, GLAST, GLT-1 and EAAC1 in the DLS 1 day after a single cocaine injection, respectively. Note the decreased immunolabeling of the cocaine-treated groups in the GFAP (B vs. A) and GLAST (D vs. C) as compared to the vehicle-treated groups.


Vehicle-Treated
Cocaine-Treated
GFAP
GLAST
GLT-1
EAAC1
A
B
C
D
E
F
G
H

vehicle-treated rats $t(46) = 2.17$, $p < 0.035$. However, glial glutamate transporter GLT-1 immunolabeling was not significantly changed in cocaine-treated rats $t(46) = 0.81$, $p < 0.42$ and neuronal glutamate transporter EAAC1 immunolabeling was slightly elevated as compared to its control group $t(74) = 0.42$, $p < 0.67$.

Discussion

In this study we investigated the role of the motor thalamus, astrocytes, and glutamate transporters in the increase in extracellular glutamate in the dorsolateral striatum 1 day after a single cocaine injection. We found that an electrolytic lesion of the motor thalamus reduced extracellular glutamate in the cocaine-naïve rat. Furthermore, the motor thalamic lesion blocked the increase in striatal glutamate 1 day after a single cocaine treatment. Interestingly, both GFAP and GLAST immunolabeling decreased in the rats exposed to the acute cocaine injection the day before. The decrease in GFAP and GLAST was small but statistically significant. Taken together, these data showed that a single 15 mg/kg cocaine injection was sufficient to increase extracellular glutamate 1 day later and that the motor thalamus, glial cells, and glutamate transporters were all significantly affected 1 day after that single cocaine treatment.

The Motor Thalamus & Acute Cocaine

Direct input to the dorsolateral striatum from the thalamus exists, however, this projection originates from the parafascicular nucleus (Sidibe et al., 2002). The projections from the parafascicular nucleus form synaptic contacts with dendritic shafts, which differ significantly from corticostriatal terminals that form synaptic contacts onto spines (Dube et al., 1988;Smith and Bolam, 1990). We have reported that less than 10% of the excitatory contacts are made onto shafts, suggesting that the thalamostriatal projection is minimal in the rat (Meshul et al., 1999). This implies that the motor thalamus does not have a direct influence on the dorsal striatum and that the main input to the dorsal striatum is indirect via the thalamocortical projection. The motor thalamus was chosen for the lesion site for this reason. Additionally, a

lesion of the motor cortex would remove the majority of the dorsal striatal glutamate, making extracellular detection difficult. By lesioning the motor thalamus, the thalamic projection to the motor cortex was eliminated, thereby reducing the indirect input from the thalamus to the dorsal striatum.

The motor thalamic lesion was bilateral to account for the ipsilateral and contralateral projections from the cortex to the striatum (McGeorge and Faull, 1989;Carman et al., 1965;Webster, 1961;Wilson, 1987). In the motor thalamic lesioned rats, the mammillothalamic tract was often lesioned because it is immediately medial to the motor thalamus. It is possible that the motor thalamic lesion could extend to other areas outside of the motor thalamus. Dorsal to the motor thalamus (VM) is the ventrolateral nucleus of the thalamus, which also projects to the sensorimotor cortex. Lateral to the motor thalamus is the sensory thalamic nuclei, which consists of the ventroposterior lateral (VPL) and ventroposterior medial (VPM) nuclei. Ventral to the motor thalamus is the zona incerta. None of the areas surrounding the motor thalamus have connections to the dorsolateral striatum or to the sensorimotor cortex, except for the ventrolateral nucleus, which is part of the motor thalamus. A previous report showed an increase in extracellular glutamate in the dorsolateral striatum after GABA was injected into the VM nucleus (Meshul et al., 1996). However, when GABA was injected into the adjacent somatosensory (VPM/VPL) nuclei of the thalamus, there was no change in striatal glutamate. Therefore, any damage to the areas surrounding the VM nucleus should not have altered the results.

Causes of Elevated Glutamate

We previously reported that the levels of extracellular glutamate decrease 28% after elimination of calcium from the aCSF in the rats injected with cocaine 1 day previously (McKee and Meshul, 2005). The current study showed that the motor thalamic lesion reduced cocaine-induced glutamate levels by 39%, which is equivalent to that of the non-cocaine treated, VM-lesioned rat. In combination, these data suggest that 30-40% of extracellular glutamate levels

are regulated by calcium-dependent, neuronal mechanisms of release. However, changes in vesicular glutamate immunolabeling as measured by quantitative immunogold electron microscopy revealed no significant changes. Therefore, it is unlikely that changes in vesicular glutamate caused the cocaine-induced decrease in extracellular glutamate in the dorsolateral striatum (McKee and Meshul, 2005). For this reason, we did not suspect that the vesicular glutamate transporter (VGLUT) played a role in extracellular glutamate alterations because the relative density of nerve terminal glutamate immunolabeling was unchanged 1 day after a cocaine injection. Hence, changes in the relative density of VGLUT immumolabeling were not measured.

Astrocytes and Glutamate Transporters

It was surprising that no changes in GLT-1 were found after acute cocaine exposure. GLT-1 is the predominant glutamate transporter in the striatum and GLAST immunoblots account for very low levels of GLAST in the striatum (Rothstein et al., 1994). However, the ultrastructure shows GLAST-positive astroglial processes (Rothstein et al., 1994). Inhibition of 84% of striatal GLAST with antisense oligonucleotides was associated with a 35% loss in glutamate transport (Rothstein et al., 1996). The glial transporters GLT-1 and GLAST account for approximately 80% of glutamate transporter in the striatum (Rothstein et al., 1996). This contrasts with a report suggesting that greater than 90% of glutamate transport can be accounted by GLT-1 alone (Haugeto et al., 1996). The predominance of GLT-1 helps explain why the change in immunolabeling for GLAST is small, albeit significant.

These experiments did not determine if the sodium-independent cystine/ glutamate transporter contributed to the cocaine-induced increase in extracellular glutamate in the dorsolateral striatum. The antiporter studies carried out by Kalivas and colleagues have focused largely on the ventral striatum, which receives glutamatergic afferents from limbic and association cortices and dopaminergic afferents from the ventral tegmental area (Baker et al., 2002). In fact, (S)-4-carboxyphenylglycine (CPG), which was used to block the

cystine/glutamate transporter (Baker et al., 2002), also antagonizes group I metabotropic glutamate receptors, and is an agonist of group II metabotropic glutamate receptors. Therefore it is possible that the antiporter and metabotropic glutamate receptors also regulate cocaine-stimulated extracellular levels of glutamate in the dorsolateral striatum. In the dorsolateral striatum, we found that the decrease in the extrasynaptic Na^+ dependent glutamate transporter GLAST was inversely associated with an increase in cocaine-stimulated extracellular glutamate levels.

There are no changes in GFAP immunolabeling in the dorsal striatum following withdrawal from 7-daily repeated cocaine injections (Bowers and Kalivas, 2003). However, we are unaware of any reports that show changes in GFAP immunolabeling in the dorsolateral striatum following acute cocaine administration. GFAP is only a marker for mature astrocytes and will not detect changes in other types of glial cells or immature astrocytes. Although astrocytes are only one type of glial cell, GFAP immunolabeling has been shown to change in response to plasticity (Sirevaag and Greenough, 1991). It is particularly interesting that changes in GFAP and GLAST immunolabeling both developed in response to an acute cocaine injection 1 day later. A decrease in GFAP immunolabeling suggests a diminished surface area for GLAST localization. Although no alterations in GLT-1 were found, it is possible that the decreased immunolabeling of GFAP and GLAST are related. The cocaine-induced increase in extracellular glutamate could be caused by a decrease in the Na^+ dependent glutamate transporter activity. This would especially be interesting to test in light of the decreased GLAST immunolabeling found 1 day after a single injection.

Conclusions

This study provides evidence for neuronal and glial processes in the development of increased extracellular glutamate in the dorsolateral striatum 1 day after a single cocaine injection. The increase in striatal glutamate is mediated by the thalamocortical input to the dorsolateral striatum, and also by diminished astrocyte and glial glutamate transporter density.

It is possible that these changes contribute to psychostimulant-induced motor patterns, such as stereotypic behavior. A single dose of cocaine is sufficient to induce long lasting changes that affect the basal ganglia and may relate to plastic events that predispose the subject to drug-related behaviors.

These experiments demonstrate that the increase in striatal glutamate 1 day after cocaine occur via the basal ganglia circuitry and are associated with changes in glial cells and glial glutamate transporters. What is not clear is the mechanism for the decrease in extracellular glutamate 3 days after a single injection of cocaine. It is unlikely that the motor thalamic lesion experiment would delineate a role for the thalamocortical pathway in the 3-day decrease in striatal glutamate. A lesion of the motor thalamus decreases striatal glutamate in the vehicle-treated rat. The combination of the motor thalamic lesion and cocaine treatment would significantly decrease striatal glutamate 3 days later. The ability to dissociate the decrease in striatal glutamate from the lesion and the decrease in striatal glutamate from the cocaine treatment would make the results difficult to interpret. Additionally, the decrease in striatal glutamate resulting from a single cocaine injection is present at 3 days and lasts for at least 2 weeks. It is likely that there are changes in the dorsolateral striatum that could account for these long lasting neurochemical changes. Glia cells are uniquely positioned in their ability to maintain a long lasting decrease in striatal glutamate. Glia cells release glutamate and take up glutamate from the extracellular space via glutamate transporters. The decrease in striatal glutamate could be associated with an increase in the glutamate transporters that take up glutamate from the extracellular space. There could be an increase in glutamate transporters available for glutamate reuptake, an increase in the glial cells that the glutamate transporters are located on, or both. Therefore, it is hypothesized that the 3-day decrease in striatal glutamate is associated with an increase in astrocytes and glutamate transporters and that antagonism of the glutamate transporters could block the cocaine-induced decrease in striatal glutamate.

Chapter 3

Intrastriatal microinjection increases glial fibrillary astrocytic protein (GFAP) immunoreactivity in the rat dorsolateral striatum

Abstract

We have reported that a single systemic injection of cocaine produces a significant decrease in extracellular glutamate in the dorsolateral striatum of the rat 3 days after that acute cocaine exposure (McKee and Meshul, 2005). This decrease was not associated with alterations in the density of glutamate immunolabeling in identified striatal nerve terminals. We hypothesized that the cocaine-induced decrease in extracellular glutamate could be due to the effects of cocaine on glial cells and the glutamate transporters located on them. The present study investigated the response of glial cells and sodium-dependent glutamate transporters 3 days after a single cocaine exposure. First, the relative density of the glia structural protein marker, GFAP, and glutamate transporters GLT-1, GLAST, and EAAC1 was investigated 3 days after a single cocaine injection using immunohistochemistry. Cocaine treatment did not alter the density of immunolabeling of these markers as compared to the control groups. Next, we hypothesized that cocaine would differentially activate the glutamate transporters, which could alter the levels of extracellular glutamate. Glutamate transport was antagonized by infusing L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) into the dorsolateral striatum just prior to the systemic cocaine injection. The PDC-infusion increased striatal extracellular glutamate in the cocaine treated rats beyond the levels found in the control group. However, the microinjection of artificial cerebral spinal fluid (aCSF) blocked the cocaine-induced decrease in striatal glutamate. This finding suggested that the damage from the microinjection was sufficient to increase striatal glutamate levels without the presence of the PDC. Therefore, the damage from the microinjection had to be taken into consideration when assessing changes in extracellular glutamate. To investigate the damage caused by the microinjection, GFAP immunolabeling studies were carried out on tissue that was microinjected with aCSF or PDC three days previously. GFAP immunolabeling was not changed in the dorsolateral striatum (DLS) among the microinjected groups. However, GFAP immunolabeling in the dorsolateral striatum was

significantly increased compared to the ventromedial striatum in the groups that received the aCSF microinjection. Increased GFAP immunoreactivity suggests that the damage from the microinjection was sufficient to disrupt the ability to effectively measure extracellular glutamate.

Introduction

Sodium-dependent glutamate transporters regulate extracellular levels of glutamate by rapid removal of glutamate from the extracellular space (Nicholls and Attwell, 1990). Three glutamate transporters have been identified in the rodent: Excitatory amino acid transporter 1, 2, and 3 (EAAT1,2,3). Glutamate/ aspartate transporter (GLAST or EAAT1) and glutamate transporter-1 (GLT-1 or EAAT2) are located on glial cells and remove the majority of glutamate from the extracellular space into the glial cells after stimulation. The excitatory amino acid carrier 1 (EAAC1 or EAAT3) transporter is located on the postsynaptic spines and thereby increases glutamate within the postsynaptic cell. Inhibiting GLAST and GLT-1 with specific antisense deoxyoligonucleotides increases extracellular glutamate (Rothstein et al., 1996). However, extracellular glutamate is not increased by inhibition of EAAC1.

Antagonizing the glutamate transporters can increase extracellular glutamate. The glutamate transporter antagonist, L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), is a substrate of the glutamate transporters that competitively inhibits glutamate uptake and leads to a reversal of the glutamate transporters (Bridges et al., 1991). PDC selectively binds to the glutamate transporters and does not bind to glutamate receptors (Bridges et al., 1991). Reverse dialysis of PDC leads to an increase in extracellular glutamate in the striatum (Segovia et al., 1997;Rawls and McGinty, 1997) and this increase is dependent on calcium and sodium channels (Rawls and McGinty, 1997).

Glial fibrillary acidic protein (GFAP) is a structural protein in mature astrocytes and is used as a marker to detect changes in mature astrocyte proliferation (Buckman and Meshul,

1999;Bowers and Kalivas, 2003). Withdrawal from repeated cocaine administration does not alter GFAP-positive cell counts in the dorsal striatum (Bowers and Kalivas, 2003), however the changes in GFAP labeling after acute cocaine exposure are not known. Astrocyte proliferation can increase glutamate removal from the extracellular space. A proliferation in astrocytes could increase the number of glutamate transporters available to remove glutamate from the synaptic cleft. This would lead to an increase in the density of glutamate transporters as well. Interestingly, decreases in GLT-1 immunolabeling after treatment dopamine depletion with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is associated with an increase in GFAP immunolabeling (Dervan et al., 2004).

Considerable attention has been paid to the cocaine-induced changes in glutamatergic systems in the past decade (Herman et al., 2002). What is becoming clear is that cocaine can induce long-term changes in the glutamate system in the basal ganglia after an acute or repeated exposure. Recently we reported that 3 days after a single injection of cocaine, extracellular glutamate within the rat dorsolateral striatum decreases significantly as compared to controls (McKee and Meshul, 2005). This cocaine-induced decrease in extracellular glutamate was not associated with alterations in nerve terminal glutamate immunolabeling in the dorsolateral striatum. Extracellular glutamate levels in the cocaine-treated rats were reduced by 35% after calcium was removed from the aCSF perfused through the microdialysis probe. Extracellular glutamate levels in the cocaine-treated rat are mediated by neuronal (i.e. calcium-dependent) release of glutamate in part. The possibility remains that there is also a glial component as well. An increase in the density of glutamate transporters or astrocytes could lead to a decrease in extracellular striatal glutamate. In addition, the glutamate transporters could have increased activation, which could lead to a decrease in extracellular glutamate in the dorsolateral striatum.

Therefore it was hypothesized that the density of GFAP, GLT-1, GLAST, and EAAC1 would be increased 3 days after a single injection of cocaine. Furthermore, it was hypothesized

that antagonism of the glutamate transporters with PDC would block the cocaine-induced decrease in striatal glutamate. To investigate the role of glutamate transporters in the cocaine-induced changes in extracellular glutamate, the relative density of GFAP, GLT-1, GLAST, and EAAC1 immunolabeling was measured three days after acute cocaine administration. To determine if the decrease in extracellular glutamate was caused by increased glutamate transporter activity, PDC was microinjected into the dorsolateral striatum just prior to a systemic cocaine injection. Three days later, dialysate samples from the dorsolateral striatum were collected.

No alterations in the relative density of GFAP or the glutamate transporter immunolabeling were found 3 days after a single cocaine injection. PDC elevated extracellular glutamate compared to the control group. Interestingly, the aCSF microinjection masked the cocaine-induced decrease in striatal glutamate. This masking was evident by the increase in GFAP immunolabeling in the dorsolateral striatum as compared to the ventromedial striatum in the aCSF-microinjected group.

Methods

Subjects

Male Sprague-Dawley rats (3 months old, Harlan, Indianapolis, IN) were housed at the Veterinary Medical Unit at the Veteran's Administration Medical Center in Portland, Oregon. Rats were housed in pairs in clear plastic cages with corncob bedding. Animals were maintained in a temperature and light controlled room on a 12-hour light/ dark cycle, with access to food and water ad libitum. All animals were drug and experimentally naïve at the beginning of the experiments. Care was taken to minimize the number of animals used and to reduce animal discomfort. Examples of these modifications include implantations of double guide cannulae to use less rats and the use of the post-operative analgesic, buprenorphine. All

procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Veteran's Affairs Medical Center's Institutional Animal Care and Use Committee (IACUC).

Drugs

Cocaine hydrochloride (Coc) (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water or 0.2 ml sterile water for vehicle-treated (Veh) animals intraperitoneally. The cocaine was made each day just prior to use. L-trans-pyrrolidine-2,4-dicarboxylate (PDC) (Tocris, Ellisville, MO) was diluted into sterile aCSF for microinjections and stored at 4°C.

Cannula Implantation

Rats were anesthetized with isoflurane (1.5 – 4%) and received air at all times during the procedure (1-2% oxygen). Their heads were shaved and placed in a Stoelting stereotaxic apparatus. Their skulls were exposed by a small incision made on the skin covering their heads and 1 or 2 holes were drilled using a dental drill into the skull depending on the following criteria. The rats used in the microdialysis experiment were implanted with a single guide cannula above either the left or the right hemisphere. In the microinjection experiment where GFAP immunolabeling was carried out, all rats were implanted with two guide cannulae above the left and right dorsolateral striata. The guide cannulae were placed above the dorsolateral striata using the following coordinates: (mm from Bregma) ±0.5 rostral and ±3.1 lateral (Paxinos and Watson, 2005). Three additional holes were drilled for rats receiving a single guide cannula and 2 additional holes were drilled for rats that received 2 guide cannulae. Dental screws were placed in the holes. A stainless steel guide cannula (15 mm long, 20-gauge) (Small Parts, Miami Lakes, FL) was lowered 0.5 mm from the surface of the skull without penetrating the dura. Dental cement was applied to the base of the guide cannula and the flathead screws to stabilize the guide cannula assembly. Epoxy was added on top of the dental cement to further stabilize the assembly. Then the skin around the assembly was superglued and stapled and the rats were allowed to recover for at least five days.

Microinjection

The rats were habituated by daily handling and placement of a modified microinjector into the guide cannula so that the microinjector did not pass the bottom tip of the guide cannula. This handling was done everyday for 1 week prior to the experiment and the rats were held for 5 minutes each. A modified stylet was inserted that extended only the length of the guide cannula and was inserted during the handling.

The microinjectors were constructed by welding a 26-gauge piece of stainless steel tubing inserted into 20-gauge stainless steel tubing. The 26-gauge tubing extended 17.5 mm beyond the 20-gauge piece. A silica glass fiber (0.15 mm outer diameter, 0.07 mm inner diameter, Polymicro Tech) was inserted into the 26-gauge fiber so that it extended 2 mm beyond the end of the 26-gauge tubing. The silica was secured to the tubing with super glue. The microinjector reached into the dorsolateral quadrant of the striatum, 1.5 mm below the ventral edge of the corpus callosum. This length was chosen based on initial studies where thionin stain was injected through the microinjector. It was found that the injected fluid dispersed laterally and dorsally for the 1.5 mm extent of the dorsolateral quadrant.

On the day of the experiment, the rats were gently restrained and a 26-gauge pin was used to clean the guide cannula and pass through the brain 18 mm from the top of the guide cannula. This punctured the dura and allowed the microinjector to be lowered into the dorsolateral striatum without damage to the microinjector. The microinjector was filled with aCSF containing 0, 1, or 10 nmol/ 100 nl (0 ng, 160.94 ng PDC or 1609.4 ng respectively), however, it is unlikely that the full concentration was infused because the solution could be displaced upon microinjector insertion into the dorsolateral striatum. Then, the microinjector was inserted into the guide cannula. For the microinjection study where GFAP immunohistochemistry was carried out, different concentrations of PDC were injected into the left and right hemispheres separately to reduce the number of animals used.

The 20-gauge PE tubing was completely filled with solution and was connected to a 10-μl glass Hamilton syringe. Care was taken to eliminate any air bubbles in the syringe or tubing. The syringe was secured on a Razel Pump and the volume was infused for 30 seconds at 3.33 nl/sec for each rat. The microinjector was left in the guide cannula for 2 minutes after the infusion was complete to allow time for the drug to diffuse, and then removed slowly. Twenty minutes after the microinjection, rats were injected with either 15 mg/kg cocaine or vehicle (i.p.). This time was chosen in order to give the PDC sufficient time to induce changes in extracellular glutamate and was based on the data obtained on Figure 11 and a previous PDC microinjection study (Segovia et al., 1997).

In Vivo Microdialysis

Dialysis probes were assembled according to Robinson and Wishaw (Robinson and Whishaw, 1988) with modifications (Meshul et al., 1999). The probes were 210 μm in diameter and the cellulose tips were 2 mm in length to effectively target the dorsolateral quadrant of the striatum. Probe recovery was tested by inserting the microdialysis probe into a solution of aCSF and 10 pmol/μl of glutamate. Three 10-minute samples were collected. Then aCSF containing 4 mM PDC was infused through the microdialysis probe to determine if PDC affected the ability of the probe to recover glutamate. Three 10-minute samples were collected and the aCSF was switched back to the normal aCSF. These dialysates were analyzed via HPLC for the concentration of glutamate. The ability of the probes to recover in vitro glutamate ranged from 5 to 10% and did not change after perfusion of the PDC-containing aCSF. This showed that PDC did not alter probe recovery of glutamate as compared to the normal aCSF alone.

The day before the microdialysis experiment animals were lightly anesthetized with isoflurane as described previously. A probe was carefully lowered through the guide cannula to the level of the dorsolateral striatum. The probe was secured in the guide cannula by epoxy. Artificial cerebral spinal fluid (aCSF) (140 mM NaCl, 3.36 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM

$MgCl_2$, 1.45 mM NaH_2PO_4, and 4.85 mM Na_2HPO_4, pH 7.4) was perfused through the probes at 2 μl/min for 30 minutes and then the rate was lowered to 0.5 μl/min overnight. The animals had access to food and water throughout the night.

The next morning, the aCSF flow rate was increased to 2 μl/min for 30 minutes prior to the collection of the microdialysis samples. The food and water were removed and four, 15-minute samples were collected. Schematic representations of the probe placements for the dorsolateral striatum have been shown previously (McKee and Meshul, 2005).

HPLC Detection of Glutamate

The dialysate within each vial collected was analyzed for glutamate concentrations. Glutamate concentration was determined using a Hewlett Packard HPLC 1090 and a Hewlett Packard 1046A Programmable Fluorescence Detector. Dialysates were derivatized with o-phthalaldehyde (OPA) and chromatographed as previously reported (Meshul et al., 1999;Meshul et al., 2002;Schuster, 1988). The sensitivity for glutamate was in the subpicomole range. The concentration of dialysate within each sample was separately averaged at each time point and then a grand mean determined for each group. The values are expressed as the mean ± SEM in picomoles/ μl.

Histological Verification

After the completion of the microdialysis experiment, animals were transcardially perfused with a 0.1% picric acid, 0.5% paraformaldehyde, 1M HEPES buffer, 2.5% gluteraldehyde fixative. The rostral striata were cut into 100 μm thick sections using a vibratome and stained with thionin to histologically verify the probe placement within the dorsolateral striatum. If the probe placement was not in the dorsolateral quadrant of the striatum, then the animal was excluded from the study.

Immunohistochemistry

Rats were transcardially perfused with 6 mls of 10% heparin in 0.1M phosphate buffer followed by 300 ml of acrolein fixative (1% acrolein in 2% paraformaldehyde diluted with 0.1M phosphate buffer) using a peristaltic pump (Cole Parmer, Vernon Hills). The dorsolateral striatum (from the rostral striatum to the anterior commissure) was sectioned (60 μm) with a vibratome (System 1000, Ted Pella, Redding, CA). Sections were incubated in 0.1% sodium borohydride in phosphate buffer to remove excess aldehyde groups for 30 minutes and then washed in a blocking solution containing 0.1 M phosphate buffer, 10% normal goat serum, and 0.1% Triton X-100 for 1 hour. The tissue was then incubated in the following primary antibodies overnight at 4°C. The guinea pig polyclonal primary antibody was GLAST (1:5000; Chemicon, Temecula, CA). The rabbit polyclonal primary antibodies were GLT-1 (1:30,000; Dr. David V. Pow, please see (Reye et al., 2002)) and EAAC1 (1:2500; Chemicon, Temecula, CA). GFAP (1:1000) was a mouse monoclonal antibody (Sigma, St. Louis, MO). Tissue were rinsed in Triton-X-free blocking solution and then incubated in the secondary antibody (biotinylated goat anti-rabbit, anti-mouse, or anti-guinea pig IgG diluted to 0.44% in 0.1 M phosphate buffer, 1:200) for 1.5 hours. Next the tissue was exposed to an avidin-biotin complex (Vector ABC kit) and enhanced with diaminobenzidine (DAB, Vector Peroxidase Substrate kit). The tissue was exposed to the nickel-enhanced DAB solution for 2 minutes for the GFAP and GLT-1 antibodies and 1.5 minutes for the EAAC1 and GLAST antibodies. After rinsing the tissue in 0.1 M phosphate buffer, the tissue was mounted on gelatin-coated slides. The slides were allowed to dry overnight, and then cover-slipped with Protex.

Optical Density Measurements

Images were captured directly using a Zeiss Axioplan light microscope (Carl Zeiss Inc., Germany) with a 5x objective (final magnification will be 62.5x). Relative optical density measurements were carried out using Image-Pro Plus software. The dorsolateral striatum was outlined using the Image-Pro software, and then the relative optical density value was obtained

for this area. At least five sections of the dorsolateral striatum from each rat were used to obtain optical density values. For the GFAP immunhistochemical analysis on the microinjected tissue, two areas of the rostral striatum were analyzed. The dorsolateral area was the target of the microinjection and was compared to the ventromedial area, which was furthest away from the microinjection in the striatum. These areas were outlined using the Imago-Pro software and the optical density measurements were obtained. The size of the area used to obtain optical density measurements did not vary between tissue sections or striatal regions. An example of these striatal areas is shown in Figure 16.

Statistical Analysis

In the first microdialysis experiment, the samples collected after reverse dialysis of PDC were compared to the baseline samples collected previously. Extracellular levels of glutamate were compared using one-way ANOVA test with Tukey's posthoc test for mean comparisons. In the microdialysis and microinjection experiment, the levels of extracellular glutamate were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare differences between groups. In the last microdialysis experiment, changes in extracellular glutamate 3 days after a single cocaine injection were analyzed using a Student's *t*-test. For the GFAP and glutamate transporter immunohistochemical study, the optical density value for cocaine-treated groups were compared to their respective vehicle-treated group for each antibody and significance was determined using a Student's *t*-test. The tissue was divided into half and the immunohistochemistry runs were carried out on two separate days due to the large number of sections. The optical density values were analyzed to determine if there was an effect of pass, and no effect of pass was found for GFAP, EAAC1, GLT-1, and GLAST. For the GFAP immunohistochemical experiment comparing different striatal areas, the optical density values were analyzed using a one-way ANOVA test with Tukey's posthoc test for comparison of group differences. Statistical significance was set at $p < .05$. JMP (version 5.0.1.2) was used to perform all statistical analysis.

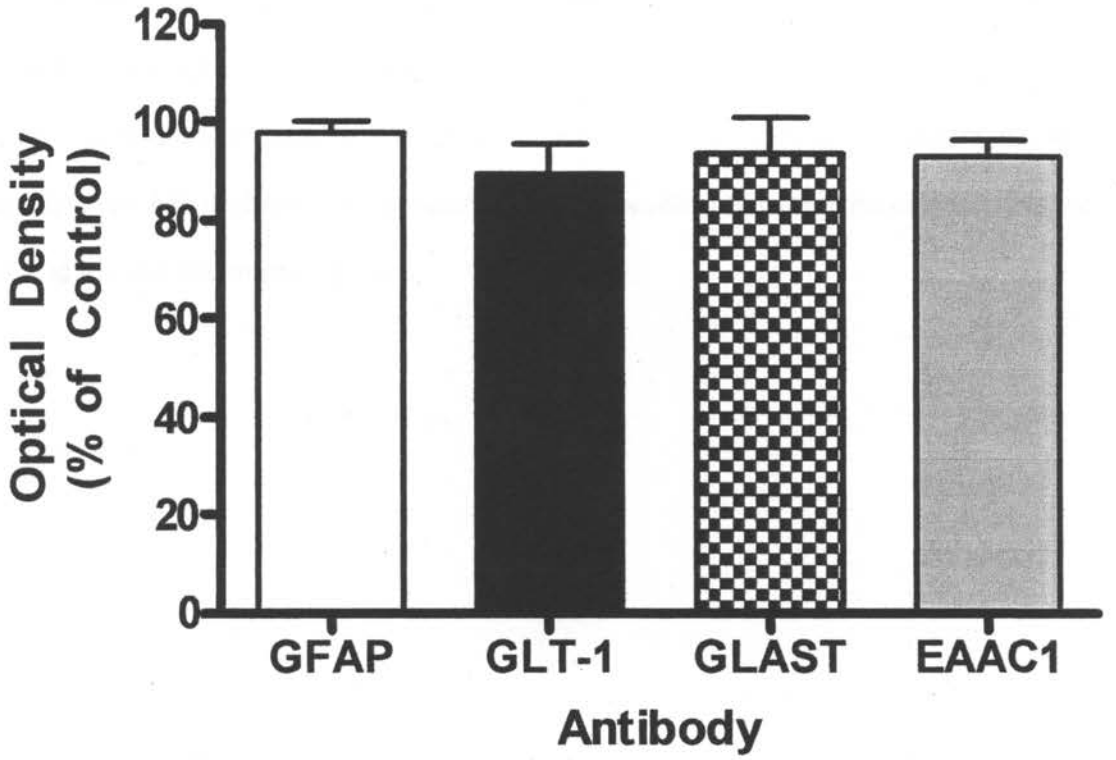
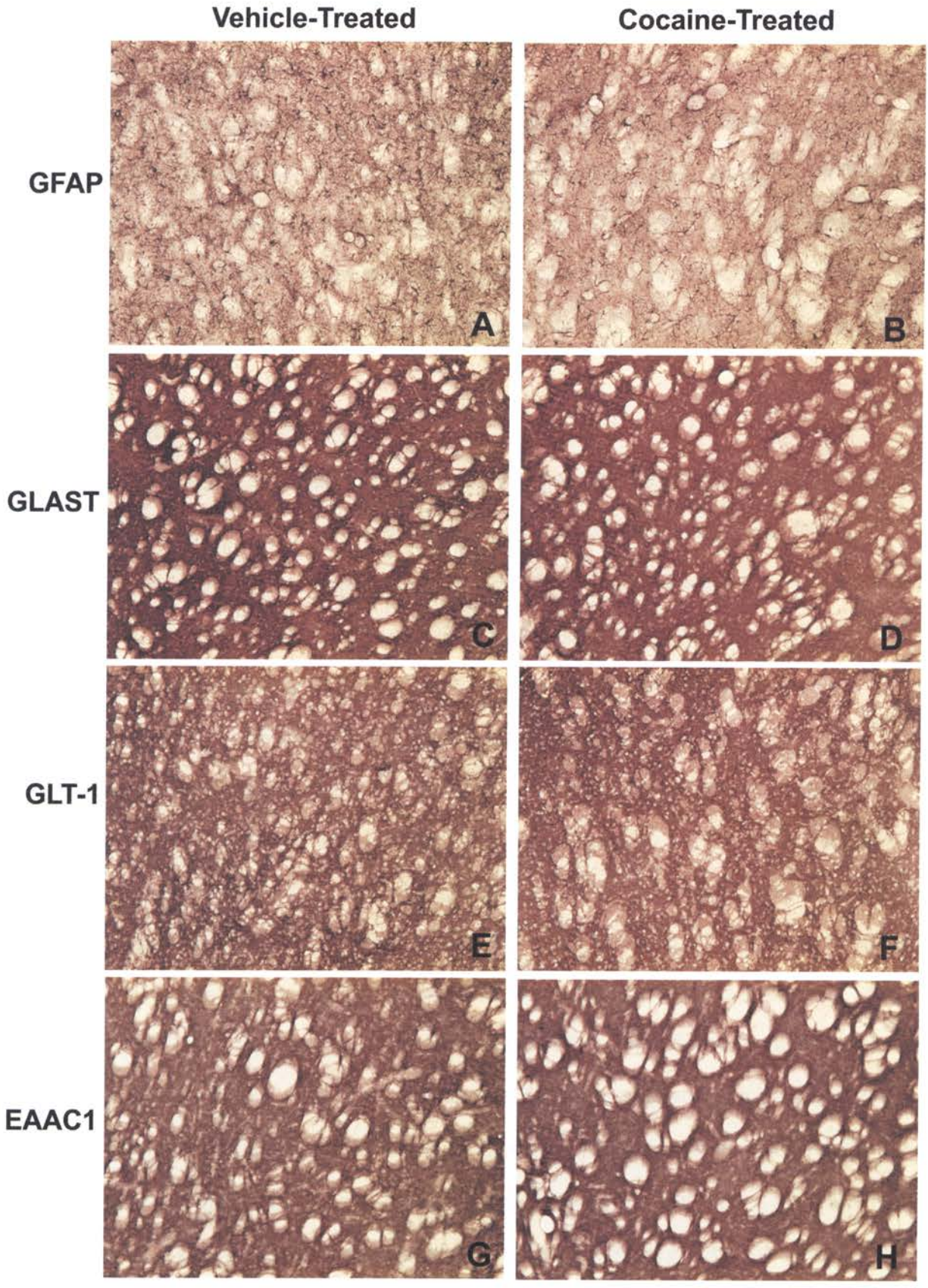


Figure 9: GFAP and glutamate transporter immunolabeling in the dorsolateral striatum was not altered three days after a single i.p. cocaine 15 mg/kg injection. Each bar represents the percent of control optical density value resulting from acute cocaine treatment (% control ± S.E.M.). Each cocaine-treated group was compared to its respective vehicle-treated group and significance was determined using a Student's *t*-test (n = 6-12).

Figure 10: Representative photomicrographs of the GFAP and glutamate transporter immunolabeling of the dorsolateral striatum of the rat 3 days after a single cocaine or vehicle treatment. A, C, E, G are photographs demonstrating the immunolabeling of GFAP, GLAST, GLT-1 and EAAC1 in the DLS 3 days after a vehicle injection, respectively. B, D, F, H are photographs demonstrating the immunolabeling of GFAP, GLAST, GLT-1 and EAAC1 in the DLS 3 days after a single cocaine injection, respectively. There was no significant difference in the density of GFAP or glutamate transporter immunolabeling between the cocaine-treated groups and the vehicle-treated groups.


Vehicle-Treated
Cocaine-Treated
GFAP
GLAST
GLT-1
EAAC1
A
B
C
D
E
F
G
H

Results

GFAP and Glutamate Transporter Immunolabeling

Immunohistochemistry was used to determine the changes in the relative density of glutamate transporters and GFAP in the dorsolateral striatum 3 days after a single 15 mg/kg i.p. cocaine injection. There is no significant change in the immunolabeling of GFAP $t(105) = 0.30$, $p > 0.78$ after acute cocaine treatment (Figure 9). There were no changes in the immunolabeling of GLT-1 $t(56) = 0.63$, $p > 0.53$, GLAST $t(72) = 0.65$, $p > 0.51$, or EAAC1 $t(120) = 1.44$, $p > 0.15$.

Microinjection and Microdialysis

To ensure that PDC increased extracellular glutamate, 4 mM PDC was infused through the probe in separate animals. This concentration was chosen because it sufficiently antagonized glutamate transporters for at least 1 hour after intrastriatal application and was not sufficient to induce toxicity (Segovia et al., 1997;Lievens et al., 1997;Massieu et al., 1995). Six baseline samples were taken, and then PDC was infused through the microdialysis probe. PDC increased extracellular glutamate by 225% for an hour and 15 minutes (Figure 11). The increase in extracellular glutamate was statistically significant 45 minutes to 90 minutes (time 135-180) after reverse dialysis of PDC $F(6,3) = 39.29$, $p < 0.007$.

To determine the role of glutamate transporters in the cocaine-induced decrease in striatal glutamate 3 days after a single cocaine exposure, rats received an intrastriatal injection of the glutamate transporter antagonist, PDC or aCSF just prior to an i.p. injection of 15 mg/kg cocaine or vehicle. Microinjections of 1 and 10 nmoles of PDC were chosen because these concentrations were not toxic and should be sufficient to increase extracellular glutamate based on previous studies and the above experiment (Segovia et al., 1997;Massieu et al., 1995). The maximum possible concentrations were 10 and 100 μM respectively, although it is unclear how much solution was displaced during microinjector insertion. Three days after the intrastriatal

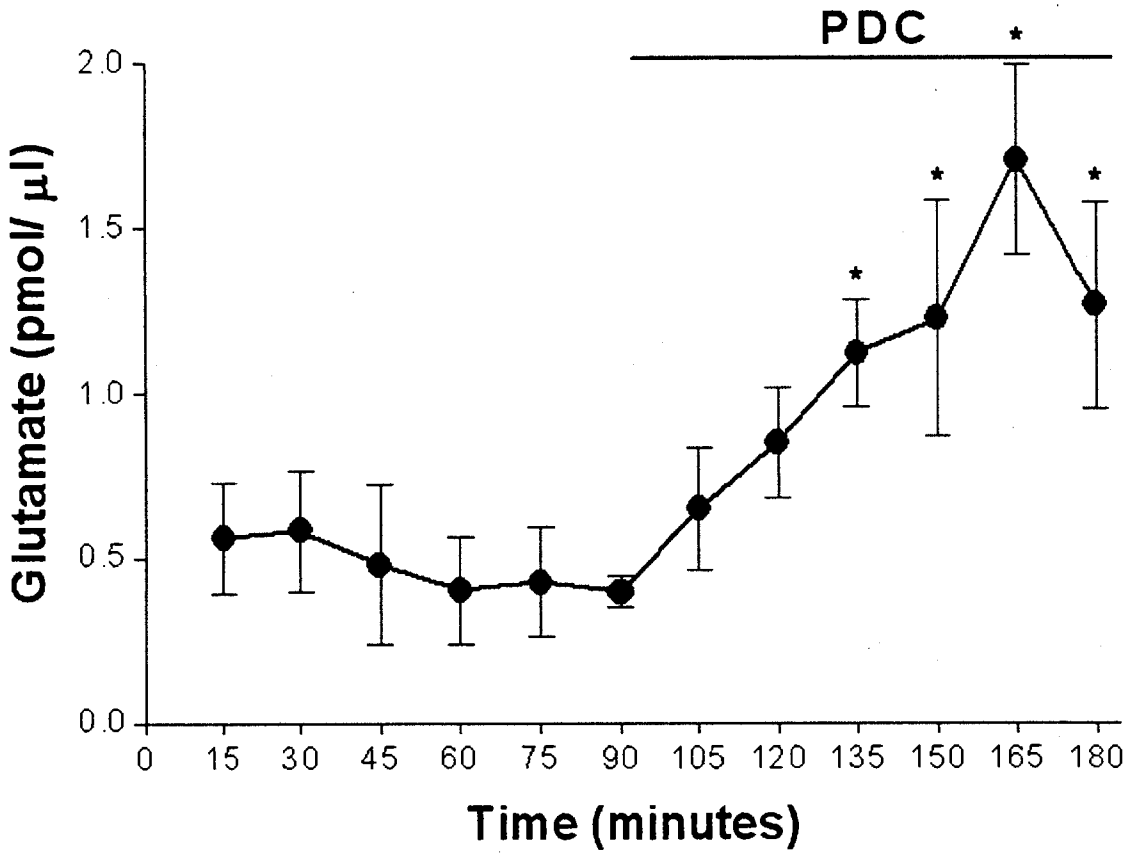


Figure 11: aCSF containing 4 mM of PDC was perfused through the microdialysis probe after collecting baseline samples in 4 rats. One-way ANOVA followed by Tukey's posthoc analysis revealed a statistically significant increase in extracellular levels of glutamate after reverse dialysis of PDC as compared to the baseline levels of extracellular glutamate (* $p < 0.05$).

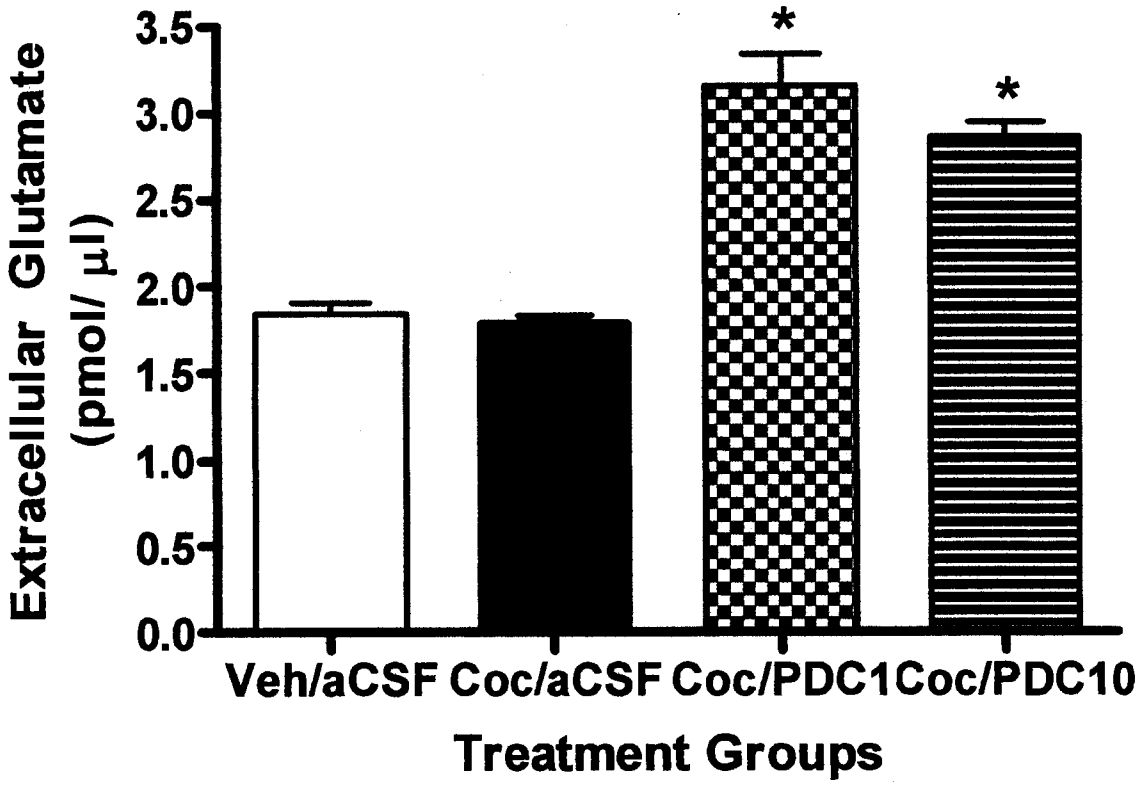


Figure 12: Rats received an intrastriatal injection of 0 (aCSF), 1 (PDC1), or 10 (PDC10) nmoles of PDC prior to an i.p. injection of 15 mg/kg cocaine (Coc) or vehicle (Veh.). Three days later, microdialysis was carried out (n = 4-6 per group). Each bar represents the levels of extracellular glutamate in pmol/µl ± S.E.M. Significance was determined using a one-way ANOVA analysis followed by Tukey's posthoc test to compare differences between groups (* = $p < 0.05$).

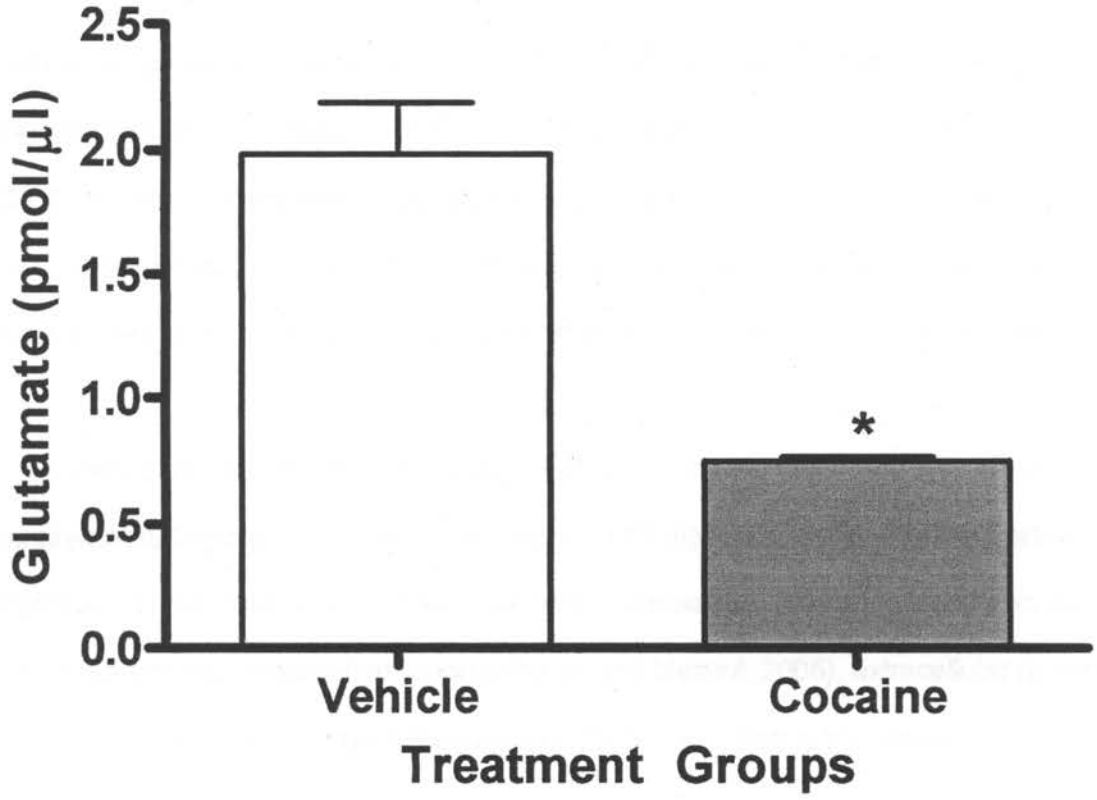


Figure 13: Extracellular levels of striatal glutamate in cocaine-treated rats were 62% lower than controls three days after a single cocaine injection (n = 5 per group). The bars represent the levels of extracellular glutamate in pmol/µl ± S.E.M. Significance was determined using a Student's *t*-test (* = $p < 0.002$).

injection of aCSF or PDC and systemic injection vehicle or cocaine, microdialysis was carried out on the four groups of rats. Figure 12 shows that the 1 and 10 nmol PDC doses increased extracellular glutamate in cocaine-treated rats 171% and 155% above that of the aCSF-microinjected groups, respectively $F(3,12) = 42.11$, $p < 0.0001$ (Coc/ PDC1 and Coc/ PDC10 vs. Veh/ Coc). However, there was no significant difference in the Coc/aCSF group as compared to the Veh/aCSF group. This contrasted with our previously published report that showed a significant decrease in striatal glutamate 3 days after a single injection (McKee and Meshul, 2005).

To show that the masking of the 3-day decrease in striatal glutamate was the result of the microinjection, 2 groups of rats were injected with 15 mg/kg i.p. cocaine with no previous microinjection. Three days later, microdialysis of the dorsolateral striatum was carried out. Similarly to our previously published report (McKee and Meshul, 2005), extracellular glutamate in rats injected with cocaine 3 days previously was 62% lower than in vehicle-treated rats $t(6) = 5.82$, $p < 0.002$ (Figure 13).

GFAP Immunohistochemistry

To determine if masking of the cocaine-induced decrease in extracellular glutamate was due to an increased glial response, GFAP immunohistochemistry was carried out 3 days after a microinjection of 0, 1, and 10 nmol PDC into the dorsolateral striatum. Optical density measurements were consistently made in the dorsolateral quadrant of the striatum. Specifically, the measurements were made lateral to the microinjection track. There were no significant differences in the optical density measurements after microinjections of aCSF, 1 and 10 nmol PDC (Figure 14) $F(3,65) = 2.05$, $p < 0.12$. Furthermore, there was no difference between the microinjected group and the levels of GFAP immunolabeling in the group that received no microinjection.

Although there was no significant difference between the microinjected groups that received the aCSF microinjection, it was noted that there was a difference between the areas

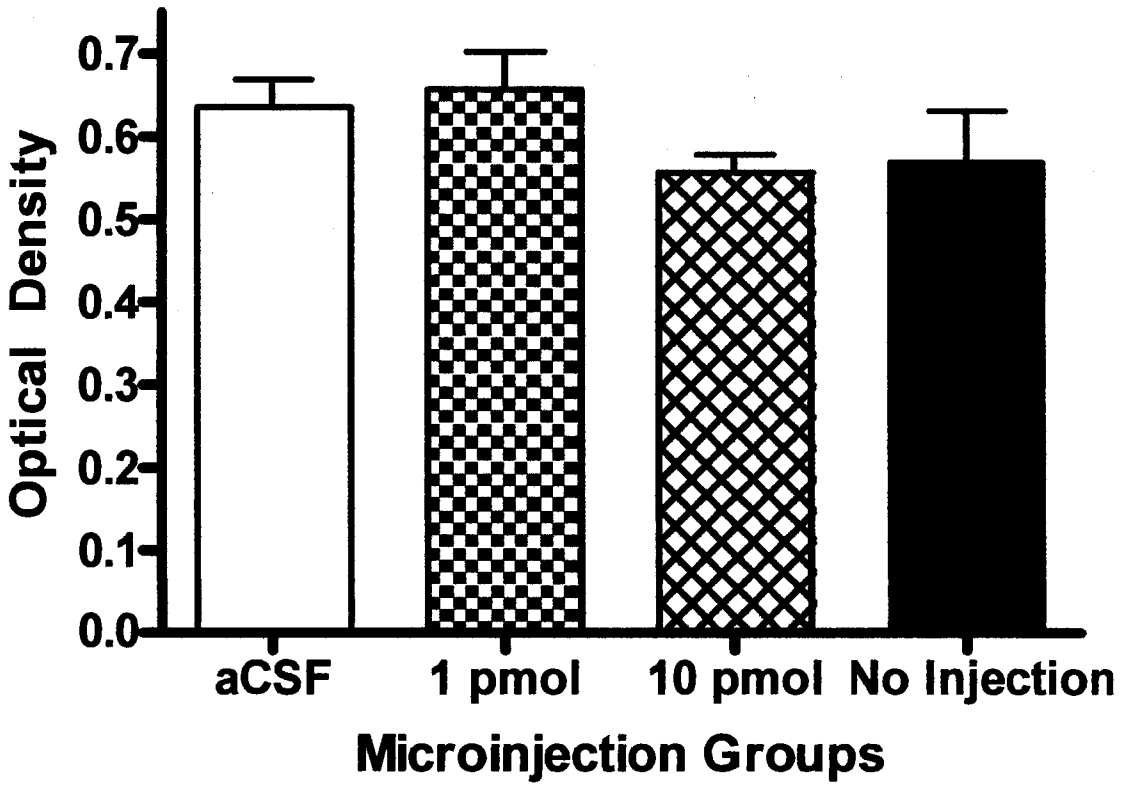


Figure 14: GFAP immunolabeling in the dorsolateral striatum was not altered three days after a microinjection of 0 (aCSF), 1 (PDC1), or 10 (PDC10) nmoles of PDC into the dorsolateral striatum. The black bar shows the optical density value for a group where the microinjector was not inserted through the guide cannula. Each bar represents the optical density value (optical density value ± S.E.M.). Significance was determined using a using a one-way ANOVA (n = 2-4).

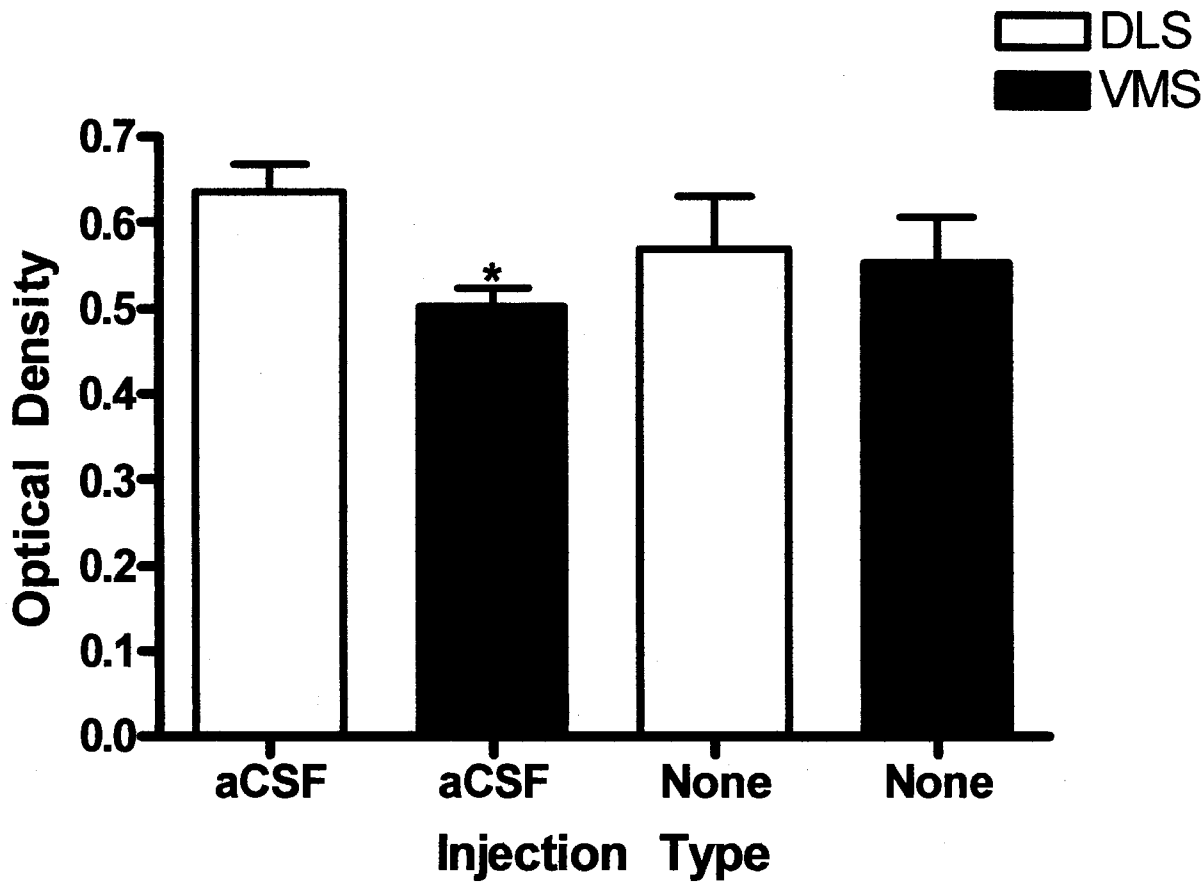


Figure 15: GFAP immunolabeling in the dorsolateral striatum (DLS, white bars) was significantly increased as compared to the ventromedial striatum (VMS, black bars) three days after a microinjection of aCSF into the dorsolateral striatum. GFAP immunolabeling was not different between the DLS and VMS in the groups that did not receive a microinjection (none). For these groups, the microinjector was not inserted through the guide cannula. Each bar represents the optical density value (optical density value ± S.E.M.). Significance was determined using a one-way ANOVA test followed by Tukey's posthoc analysis for group differences (* = $p < 0.05$, n = 2-3 per group).

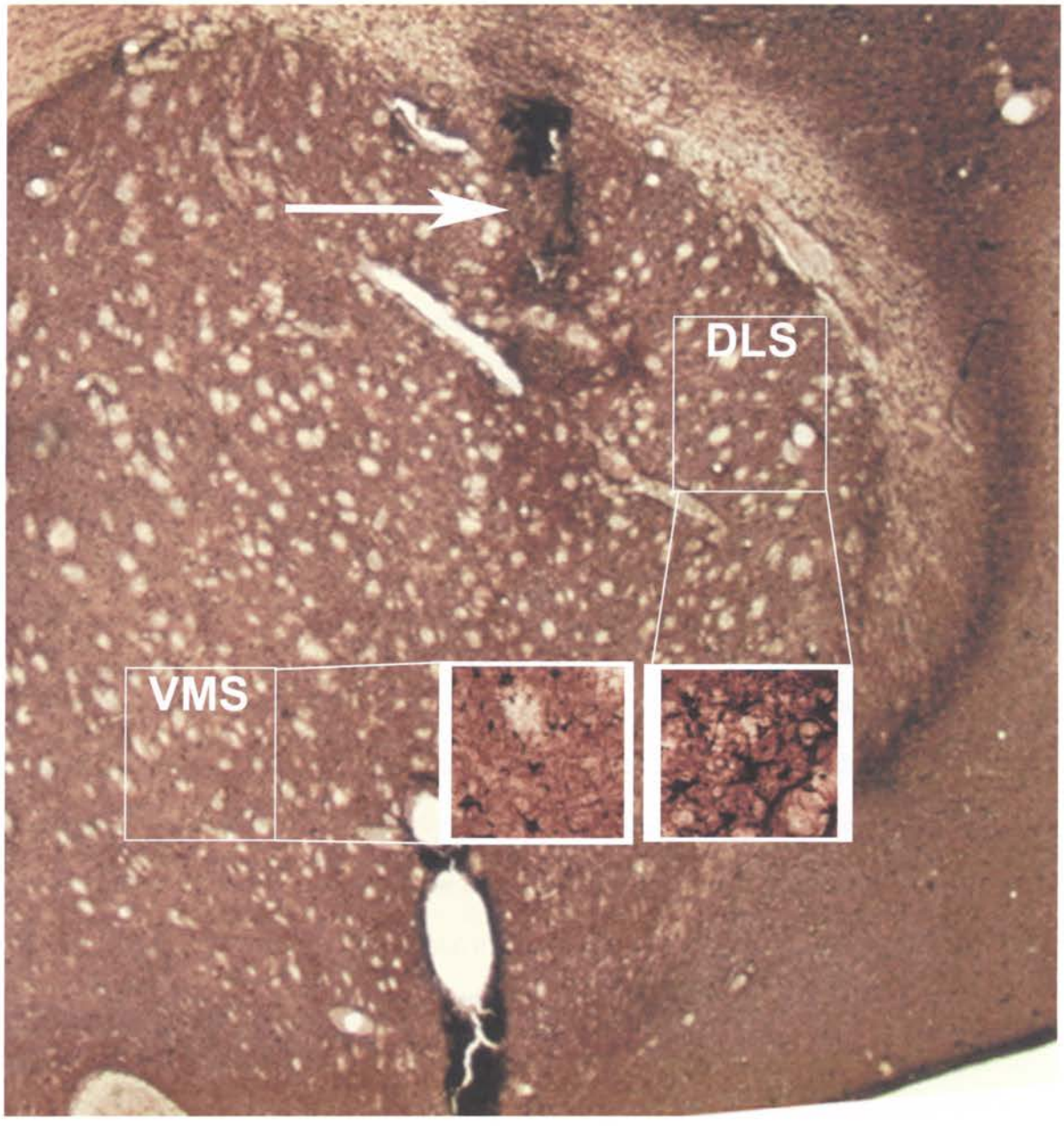


Figure 16: A representative photomicrograph of a GFAP-immunolabeled cross section highlights the differences in GFAP immunolabeling between the dorsolateral striatum (DLS) and the ventromedial striatum (VMS). The white boxes identify the regions where the optical density values were obtained and are shown at higher magnification at the lower right side of the figure. The white arrow points to the tract from the microinjector.

investigated. Optical density measurements were taken for the dorsolateral and ventromedial striatum in order to compare the area that would be affected most by the microinjection (DLS) and the area that should be affected least by the microinjection (VMS) (Figure 15). There was significant reduction in the relative density of GFAP immunolabeling between the aCSF-microinjected groups when comparing the DLS versus the VMS $F(3,54) = 3.76$, $p < 0.02$. Figure 16 is a representative micrograph that shows this difference between the DLS and the VMS. This difference was not apparent when comparing the DLS versus the VMS in the groups that had received no microinjection. Therefore, the difference between the DLS and the VMS was not due to an innate difference in GFAP immunolabeling between the two areas.

Discussion

There was no alteration in the density of GFAP and glutamate transporter immunolabeling 3 days after a single cocaine injection, suggesting that the density of transporters and astrocytes did not contribute to the reduced striatal glutamate levels. Microinjection of the glutamate transporter inhibitor PDC elevated extracellular glutamate as compared to the aCSF-microinjected group in cocaine-treated rats. Interestingly, the damage caused by the aCSF microinjection was sufficient to block the cocaine-induced decrease in striatal glutamate (Veh/aCSF vs. Coc/aCSF). The cocaine-induced decrease in striatal glutamate was demonstrated without microinjection of aCSF, suggesting that this masking of the cocaine-induced decrease was due to the microinjection itself. In addition, microinjection of 100 nl aCSF was sufficient to increase GFAP immunolabeling in the dorsolateral striatum as compared to the ventromedial striatum. This increase was not due to innate differences between the DLS and the VMS because the density of GFAP immunolabeling did not change between areas in the non-microinjected groups.

GFAP Immunolabeling

The density of GFAP immunolabeling in the dorsolateral striatum 3 days after a single cocaine injection was measured and there were no differences compared to the vehicle-treated group. GFAP is a marker for mature astrocytes only and will not indicate if other types of glial cells or immature astrocytes are proliferating as a result of cocaine exposure. Although astrocytes are only one type of glial cell, GFAP immunolabeling has been shown to change in response to plasticity (Sirevaag and Greenough, 1991) and following the loss of striatal dopamine (Dervan et al., 2004).

The density of GFAP immunolabeling in the DLS did not differ regardless of the solution injected into the dorsolateral striatum (i.e. aCSF or PDC). However, the density of GFAP immunolabeling was increased in the dorsolateral striatum following the aCSF microinjection as compared to the VMS, an area unaffected by the microinjection. Increased GFAP protein has been used as an indicator of excitotoxicity (Rothstein et al., 1996). It is possible that the increased GFAP immunolabeling indicates that the dorsolateral striatum is damaged by the microinjection. The difference in GFAP immunolabeling between the DLS and the VMS cannot be explained by innate differences in GFAP immunolabeling because there was no difference between the areas in the non-microinjected rats. These data suggest that microinjection studies that wait 3 days or longer before testing might have an increased excitotoxic factor to consider.

The density of glutamate transporter immunolabeling was unchanged 3 days after a single cocaine injection. The density of glutamate transporters did not contribute to the cocaine-induced decrease in extracellular glutamate in the dorsolateral striatum. However, transporter-mediated uptake is critical for removing glutamate from the extracellular space and preventing continued glutamate receptor activation. The unaltered density of glutamate transporter protein does not address the possibility of increased activation of glutamate transporters. An increase in glutamate transporter activation could lead to the decrease in extracellular glutamate 3 days

after a single cocaine injection. Changes in the activity of the glutamate transporters are best addressed with glutamate reuptake studies in synaptosomes (Buckman et al., 1999).

Microdialysis studies

Concentrations of PDC equal to or lower than 0.1 mM do not cause an initial increase in striatal glutamate immediately after reverse dialysis (Rawls and McGinty, 1997). In the present study, extracellular glutamate was not significantly increased until 45 minutes after reverse dialysis of PDC had commenced. This suggests that the concentration of PDC that effectively reached the tissue through the microdialysis probe was a maximum concentration of 0.1 mM.

Cocaine increases extracellular dopamine in the dorsal striatum (Hurd and Ungerstedt, 1989;Keller, Jr. et al., 1992). In addition, PDC increases extracellular dopamine in the striatum, along with GABA, and taurine (Segovia et al., 1997). The increased dopamine could bind to dopamine D-2 receptors located on corticostriatal terminals (Wang and Pickel, 2002;Cepeda et al., 2001). This would lead to an attenuation of extracellular glutamate and could explain the cocaine-induced decrease in extracellular glutamate in the dorsolateral striatum. However, since the reduction in glutamate levels does not appear immediately and takes three days to develop, it is unlikely that D2R activation could explain the attenuated glutamate.

A systemic injection of cocaine 3 days prior to microdialysis failed to produce a decrease in extracellular glutamate when aCSF was microinjected into the dorsolateral striatum as previously reported (McKee and Meshul, 2005) and shown in Figure 4A. Insertion of the microdialysis probe occurred 3 days after the intratstriatal injection, which was sufficient time for the tissue to respond to the trauma caused by the microinjection. The experimental design necessitated waiting 3 days after the microinjection to carry out the microdialysis, and it is likely that a compensatory process occurred during this time to mask the cocaine-induced decrease in striatal glutamate. Prior insertion of the microinjector would not remove this factor and is unlikely to affect the results.

Although it is unknown how the microinjection damage masked the cocaine-induced decrease in striatal glutamate 3 days, it is clear that the microinjection elevated GFAP immunolabeling in the dorsolateral striatum (Figure 15). In addition, elevated striatal glutamate in the PDC-microinjected group beyond the aCSF-microinjected group suggested that unknown processes were initiated to maintain increased extracellular levels long after the PDC is no longer present. Increased GFAP immunolabeling could be associated with increased release of glutamate from glial cells. Astrocyte proliferation could be sufficient to mask the cocaine-induced decrease in striatal glutamate found without microinjection. The combination of the tissue damage, cocaine, and PDC may have increased extracellular glutamate through a reversal of the glutamate transporters (Kanai and Hediger, 2004;Katsumori et al., 1999;Ohta et al., 2002). Also, cocaine and PDC may have reversed the cystine/ glutamate antiporter, which is important for regulating cocaine-stimulated extracellular glutamate levels in the ventral striatum (Baker et al., 2003).

Conclusions

The finding that GFAP immunoreactivity in the DLS was elevated after the microinjection as compared to the less-affected area (VMS) was especially interesting. Overall, a microinjection inhibited the ability to accurately measured extracellular glutamate in the dorsolateral striatum. Increased GFAP immunoreactivity hindered the ability to measure extracellular glutamate and may also be problematic for measuring glutamate-related behaviors such as stereotypy. This could be problematic for studies that measure a behavioral or neurochemical response days after a microinjection.

These studies did not clarify the role of the glutamate transporters in the decrease in striatal glutamate caused by a single cocaine injection 3 days later. The inability to accurately measure extracellular glutamate was caused by the microinjection damage. Therefore, the cause of the 3-day decrease remains unknown. Because the combined PDC- and cocaine-treated groups had elevated glutamate levels compared to the aCSF- and vehicle-treated

groups, it is possible that glutamate transporters contributed to the 3-day decrease in striatal glutamate.

General Discussion

The experiments described in the preceding chapters have revealed that a single systemic cocaine injection increased extracellular glutamate 1 day later in the dorsolateral striatum of the rat. Three days after a single cocaine injection, striatal glutamate was decreased. This cocaine-induced decrease in striatal glutamate persisted for 2 weeks after a single cocaine injection. These changes in extracellular glutamate were not associated with changes in the relative density of nerve terminal glutamate immunolabeling. However, the 1-day increase and 3-day decrease in striatal glutamate were 28 - 35% calcium-dependent. The increase in striatal glutamate 1 day after a cocaine injection was blocked by a prior lesion of the motor thalamus. Also, the elevated striatal glutamate levels were associated with a decrease in GFAP and GLAST immunolabeling. The decrease in striatal glutamate found 3 days after a single cocaine injection was not associated with changes in the relative density of glutamate transporter or GFAP immunolabeling. Taken together, these data suggest that both neuronal and glial mechanisms contribute to maintaining the alterations in the dorsolateral striatum that result after a single cocaine injection. However, the mechanisms that caused the transition from the increased extracellular glutamate at day 1 to the decrease in extracellular glutamate at day 3 remain unknown.

Cocaine causes an immediate increase in extracellular dopamine in the dorsolateral striatum (Church et al., 1987;Hurd and Ungerstedt, 1989). A single injection of 10 mg/kg cocaine increases extracellular dopamine for approximately 30 minutes, at which time the dopamine levels peak to 900% of controls (Church et al., 1987). After 1.5 hours, extracellular dopamine levels returned to 200% of controls. Extracellular dopamine remains elevated at 200% of control 3.5 hours after the cocaine injection (Church et al., 1987). The largest effect of cocaine on extracellular dopamine dissipates after 1.5 hours, but it is not known how long extracellular dopamine is augmented after a single cocaine injection. It has been reported that dopamine

reuptake blockade is still present up to 2 weeks after a single cocaine injection (Peris and Zahniser, 1987). From these studies, it is hypothesized that striatal extracellular glutamate could be changing in response to the heightened extracellular dopamine and dopamine reuptake blockade caused by a single cocaine injection.

Proposed mechanism for the first 0 – 2 hours after cocaine treatment

Dopamine can bind to inhibitory dopamine-2 receptors (D2R) located on the corticostriatal terminals in the DLS (Sesack et al., 1994). Activation of the presynaptic D2Rs on the corticostriatal terminals reduces the release of glutamate from those terminals. This mechanism has been suggested by studies that involve blocking increased glutamate release by application of a D2R agonist (Yamamoto and Davy, 1992;Maura et al., 1988). Yamamoto and Davy stimulated striatal extracellular glutamate to 250% above baseline by perfusing a depolarizing concentration of potassium through the microdialysis probe. Co-perfusion with the D2R agonist LY171555 completely blocked the elevated levels of extracellular glutamate (Yamamoto and Davy, 1992). Furthermore, this blockade was reversed when a D2R antagonist was perfused through the microdialysis probe with the potassium and the D2R agonist. Taken together, these studies suggest that it is possible to block an initial increase in extracellular glutamate by D2R activation. It is hypothesized that D2R activation prevents the increase in striatal glutamate that develops sometime between 2 and 24 hours after the cocaine injection because dopamine is binding to D2Rs and inhibiting glutamate release from the terminals. This could explain why there are no changes in striatal extracellular glutamate immediately following an acute cocaine injection (Table 2)(Reid et al., 1997;McKee and Meshul, 2005).

The aforementioned hypothesis suggests that there was an increase in extracellular glutamate immediately following the acute cocaine treatment but the ability to detect that increase with microdialysis was masked by the activation of presynaptic D2Rs. Since no immediate changes in extracellular glutamate after acute cocaine treatment were detected, it is possible that the glutamate transporters played a role. Glutamate transporters rapidly remove glutamate from the

extracellular space. An increase in the density of glutamate transporter protein or an increase in glutamate transporter activity could mask any immediate increases in glutamate transporter activity.

Proposed mechanism for the 2 – 24 hours after cocaine treatment

Although there is no change in extracellular glutamate immediately following acute cocaine treatment, there is a significant increase in striatal glutamate 1 day after a single cocaine injection (Figure 2). Therefore, between 2 and 24 hours, extracellular glutamate in the cocaine-treated rats increased as compared to the vehicle-treated rats. Elevated dopamine following cocaine would bind to postsynaptic D1R and D2R, and it is possible that the activation of the postsynaptic D1Rs and D2Rs overpowers the proposed elevated glutamate transporter activity and presynaptic D2R inhibition. It is hypothesized that the activation of postsynaptic D1Rs and D2Rs begins to overpower the ability of the presynaptic D2Rs or glutamate transporters to maintain normal extracellular glutamate levels during the decline in extracellular dopamine, which is 2 hours after cocaine treatment.

It is possible that the proposed elevation in glutamate transporter activity normalized decreases after the cocaine-induced peak in extracellular dopamine. In addition, the cocaine-induced increase in extracellular dopamine would be binding to postsynaptic D1R and D2R. It is hypothesized that the activation of postsynaptic D1Rs and D2Rs begins to overpower the ability of the presynaptic D2Rs or glutamate transporters to maintain normal extracellular glutamate levels during the decline of extracellular dopamine, which is 2 hours after cocaine treatment.

Dopamine binding to D1Rs would activate the striatal GABAergic afferents to the SNr/ EPN (the direct pathway; see Figure I1). The GABA increase would inhibit these areas, leading to a decrease in GABA release (disinhibition) in the motor thalamus. This would activate the thalamocortical projection to the dorsolateral striatum, and lead to an increase in dorsolateral striatal glutamate. In addition, the cocaine-induced increase in striatal dopamine could increase striatal glutamate through the indirect pathway (Figure I1). Activation of D2Rs on striatopallidal

neurons would inhibit GABA release in the GPe. This would lead to increased GABA release in the STN. The inhibition of the STN would decrease the glutamatergic output to the SNr/ EPN, which would decrease the GABA release in the motor thalamus. Therefore, activation of both the direct and indirect pathways would lead to an activation of the thalamocortical projection to the striatum, and effectively increase extracellular glutamate in the striatum. This effect starts to occur after the initial bolus increase of extracellular dopamine in the dorsolateral striatum. There are two pieces of evidence that support this hypothesis: the increased extracellular glutamate found 1 day after a single cocaine injection (Figure 2) and the ability to block the increase by lesioning the motor thalamus (Figure 6).

Proposed mechanism for the 24 – 72 hours after cocaine treatment

The effects of a single injection of cocaine on extracellular glutamate in the dorsolateral striatum are biphasic (Figure 2). Although initially there was an increase in striatal glutamate, after day 2, there was no significant difference in extracellular glutamate between the cocaine-treated and vehicle-treated groups. Three days after a single cocaine injection there was a decrease in striatal glutamate levels (Figure 2). This transition from an increase to a decrease is intriguing. Several experiments in this thesis research attempted to address how the cocaine-induced decrease in striatal glutamate occurred. The mechanism of the transition from an increase to a decrease remains unknown, as does the exact mechanism for the 3-day decrease. Three mechanisms have been proposed in this discussion to address the transition from the 1-day increase to the 3-day decrease in striatal glutamate: an indirect pathway mechanism, a D2R mechanism, and a glutamate transporter mechanism.

Removal of calcium from the aCSF perfused through the microdialysis probe decreased striatal glutamate by 28 to 35% regardless of elevated or attenuated extracellular glutamate levels (Table 1). These data suggest that in the cocaine-treated rat, striatal extracellular glutamate levels are partially calcium-dependent. These findings are in agreement with a previous report (Rawls and McGinty, 1997). However, these data do not necessarily suggest

that extracellular glutamate levels in the cocaine-treated rats are neuronal (i.e. mediated through the thalamocortical circuit). Since both neurons and glial cells release glutamate via a calcium-dependent mechanism (Newman, 2003), removal of calcium from the aCSF could inhibit glutamate release from either nerve terminals or the astrocytes. Calcium-dependent release of glutamate from astrocytes might explain why extracellular glutamate levels in the cocaine-treated rat are calcium-dependent but not associated with changes in vesicular glutamate immunolabeling (Table 3). Since extracellular glutamate levels are calcium-dependent in the cocaine-treated rat whether there is an increase or a decrease in striatal glutamate, it is unlikely that calcium-dependency contributes to the transition from elevated to attenuated striatal glutamate.

Indirect Pathway Mechanism. Since there was a decrease in striatal glutamate 3 days after cocaine treatment, it would be difficult to test the role of the thalamocortical circuit by lesioning the motor thalamus because a motor thalamic lesion alone causes a decrease in striatal glutamate (Figure 6) (Touchon et al., 2004). In fact, a role for the motor thalamus in mediating cocaine-induced changes in striatal extracellular glutamate was demonstrated by the motor thalamic lesion that blocked the cocaine-induced increase in striatal glutamate 1 day after cocaine treatment (Figure 6). However, the role of the thalamocortical pathway in the cocaine-induced decrease in striatal glutamate was not determined.

This does not preclude the thalamocortical pathway from contributing to the 3-day decrease in striatal glutamate. It is hypothesized that the thalamocortical input to the dorsolateral striatum produces the 3-day decrease in striatal glutamate resulting from cocaine treatment. This hypothesis would predict that the elevated extracellular glutamate levels 1 day after cocaine treatment would activate the excitatory glutamate receptors located on the striatopallidal neurons. Activation of the striatopallidal neurons via glutamate receptors 1 day after cocaine treatment would start to reverse the D2R-inhibition of the striatopallidal neurons. As the striatopallidal neurons continue to be activated 24 hours after cocaine treatment, the

glutamate receptor activation could overpower the D2R-inhibition, leading to an increase in striatopallidal activity. Activation of the striatopallidal neurons would lead to the accumulation of GABA in the motor thalamus via the indirect pathway. This would inhibit the thalamocortical projection to the dorsolateral striatum, and eventually lead to a reduction in striatal glutamate. If the inhibition of the motor thalamus persisted via this mechanism, this mechanism could explain why the decrease in striatal glutamate persists 2 weeks after a single cocaine injection. D1R stimulation of the direct pathway could not contribute to the cocaine-induced decrease in striatal glutamate because D1R activation would lead to an increase in striatal glutamate.

One approach to test the role of the thalamocortical pathway in the decrease in striatal glutamate 3 days after cocaine treatment would be to stimulate the motor thalamus and determine if this prevents the development of the 3-day decrease in striatal glutamate. Microinjection of a $GABA_A$ antagonist picrotoxin into the motor cortex stimulates striatal glutamate release (Canales et al., 2002). Microinjection of picrotoxin into the motor thalamus just prior to the systemic cocaine injection would block the decrease in striatal glutamate found 3 days after cocaine treatment.

D2R Mechanism. Elevated extracellular glutamate leads to an increase in extracellular dopamine in the dorsal striatum (Verma and Moghaddam, 1998). Previous research suggests that reuptake of dopamine is impaired up to 2 weeks following a single cocaine injection (Peris and Zahniser, 1987). It is hypothesized that the elevated glutamate 1 day after a single cocaine treatment causes an increase in extracellular dopamine, which binds to inhibitory D2Rs located on the corticostriatal terminals and reduces the release of glutamate from the corticostriatal terminals. This hypothesis predicts the attenuation of extracellular glutamate in the dorsolateral striatum 3 days after a single cocaine injection.

Glutamate Transporter Mechanism. One day after acute cocaine treatment, the density of GLAST immunolabeling was significantly decreased compared to the control group (Figure 7). This decrease in the relative density of GLAST immunolabeling was small although

significant. These data suggest that the glutamate transporters were being down regulated in response to the elevated extracellular glutamate levels at day 1. If there were fewer transporters available 1 day after cocaine treatment, there could be an increase in activity of the remaining glutamate transporters. It was hypothesized that the glutamate transporters were more active within the first 2 hours after cocaine treatment in order to prevent an increase in extracellular glutamate. Perhaps after that initial period, glutamate transporter activity remains elevated but is overshadowed by the circuit changes that lead to increased striatal glutamate at day 1. Although there were no changes in the relative density of glutamate transporters found 3 days after a single cocaine injection (Figure 9), it is not known if glutamate transporter activity is altered. It is hypothesized that glutamate transporter activity remained elevated immediately after cocaine treatment or increased in response to decrease transporter protein levels. This would lead to increased glutamate reuptake from the extracellular space, which would decrease extracellular glutamate. This mechanism could contribute to the transition from the 1-day increase to a 3-day decrease in extracellular glutamate in the dorsolateral striatum.

Final conclusions

Overall, a single injection of cocaine causes time-dependent, long lasting changes in extracellular glutamate in the rat dorsolateral striatum that involved both neuronal and glial mechanisms. The basal ganglia circuitry and glutamate transporters mediate these biphasic changes in striatal glutamate. In particular, the long lasting decrease in striatal glutamate may be of particular relevance to the development of drug-related behaviors and the predisposition of addiction.

The decrease in extracellular glutamate 3 days after a single cocaine injection equates to a silencing of the corticostriatal projections. This glutamate input from the sensorimotor cortex is critical for driving the output from the striatum. Inhibition of the corticostriatal projections essentially silences the basal ganglia, which implements adaptive motor behaviors (Graybiel, 2004). In addition, the inhibited basal ganglia enable a predominance of the

mesolimbic pathway, which is important for the ritualistic, inflexible behaviors such as stereotypy (Canales and Graybiel, 2000). In fact, this exact mechanism of silencing the basal ganglia and activating the mesolimbic pathway has been proposed to explain how stereotypic behavior develops (Canales and Graybiel, 2000). Furthermore, this mechanism has also been proposed to explain how habit formation develops after repeated psychostimulant administration (Canales, 2005). The results found in the experiments contained in this dissertation may contribute to the understanding of how motor stereotypies develop and how these behaviors can lead to the development of habits.

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