Dysregulation of System Xc- and Its Impact on Cocaine Seeking

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DYSREGULATION OF SYSTEM X_C- AND ITS IMPACT ON COCAINE SEEKING

by

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ABSTRACT
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Abnormal glutamate signaling in the brain, particularly in the nucleus accumbens core, contributes to compulsive cocaine-seeking behavior. In the nucleus accumbens, two distinct pools of glutamate exist. The synaptic pool stimulates excitatory postsynaptic receptors, leading to cocaine seeking. Cystine-glutamate exchange by system x_c^- contributes to the extrasynaptic pool and regulates neurotransmission by activating extrasynaptic receptors including inhibitory Group II metabotropic glutamate receptors, positioning this as an important mechanism for regulating nucleus accumbens activity. We and others have found decreased levels of cystine-glutamate exchange in animals withdrawn from chronic cocaine administration. In chapter 1, we describe studies directly implicating diminished system x_c^- activity in abnormal glutamate signaling necessary for promoting drug seeking. Given that altered system x_c^- activity is essential to drug seeking, understanding the cellular basis of reduced cystine-glutamate exchange would advance our knowledge of the neural basis of addiction. Unfortunately, little is known about how the system is regulated. To address this, we sought to define conditions that permit the study of system x_c^- (chapter 2). In chapter 3, we examined potential regulation of x_c^- by dopamine, nonspecific dopamine agonists, D1-liked dopamine receptor agonists, and D2-like dopamine receptor agonists. While we found D1-like receptor regulation of cystine-glutamate exchange in mixed cortical culture, we did not observe this effect in tissue punches; thus, it is unlikely that abnormal regulation of system x_c^- by dopamine underlies reduced cystine-glutamate exchange in cocaine-withdrawn animals. In chapter 4, we examined pituitary adenylyl cyclase activating polypeptide (PACAP) because it has been recently shown to regulate extracellular glutamate homeostasis. We found acute and tonic regulation of cystine-glutamate exchange by PACAP-induced stimulation of Ca^{2+}-dependent protein kinase (PKC) activity. More importantly, we also determined that PACAP regulation of system x_c^- is diminished in animals withdrawn from cocaine self-administration. These findings suggest dysregulated PACAP neurotransmission in cocaine-withdrawn animals contributes to the neural basis of addiction. By revealing novel mechanisms contributing to addiction, scientists can identify novel targets for therapeutic treatment.

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ABBREVIATIONS

System x_c^-	-	Cystine-glutamate exchange
System X_{AG}	-	Aspartate, Glutamate, and Cysteine transporter
PKA	-	cAMP-dependent protein kinase
PKC	-	Ca^{2+}-dependent protein kinase
CPG	-	(S)-4-carboxyphenylglycine, inhibitor of system x_c^-
SSZ	-	Sulfasalazine, potent selective inhibitor of system x_c^-
D-Asp	-	D-aspartate, inhibitor of system X_{AG}
dBcAMP	-	dibutyryl cAMP, cAMP analog and activator of PKA
PMA	-	Phorbol 12-myristate 13-acetate, selective PKC activator
NPPB	-	5-Nitro-2-(3-phenylpropylamino)benzoic acid, selective chloride channel blocker
NAC	-	N-acetylcysteine, cysteine prodrug that stimulates cystine-glutamate exchange
PACAP	-	Pituitary adenylyl cyclase activating polypeptide

INTRODUCTION

Overview

The development of cocaine addiction is a result of neuroplasticity that leads to compulsive use. The onset of cocaine use is due to a hedonic sensation when consumed in enough quantities leads to tolerance, elevated levels of consumption, and eventually addiction [for review, see (Hyman and Malenka, 2001)]. However, a compulsive pattern of drug taking emerges after chronic use that is thought to be the direct result of drug-induced plasticity (Hyman and Malenka, 2001, Pierce and Vanderschuren, 2010). Even after extended withdrawal and abstinence from cocaine, people maintain a high vulnerability to relapse (O'Brien et al., 1992, O'Brien et al., 1998). To date, there exists a lack of effective clinical therapeutic treatments for cocaine addiction. To identify effective therapeutics for preventing relapse, scientists must first reveal cocaineinduced neuroplasticity that contributes to addiction and relapse susceptibility.

Cocaine-induced neuroplasticity affects most of the brain, and can lead to compulsive drug seeking behavior (Hyman and Malenka, 2001, Pierce and Vanderschuren, 2010). In order to focus studies on brain regions relevant to cocaine seeking, the motive circuit is given serious attention. This is due to the fact that the nucleus accumbens core of the motive circuit is the primary output region for this circuitry. Investigators originally studied cocaine-induced long-term changes in dopamine signaling in the nucleus accumbens core, this given that cocaine is primarily viewed as a dopamine transporter blocker (Wise, 1984, Bradberry and Roth, 1989). However, previous studies have found that during the reinstatement phase of the self-administration model of addiction, described in detail below, not dopamine but rather glutamate signaling in the nucleus accumbens core is necessary for reinstatement

(Cornish and Kalivas, 2000). Though glutamate is necessary for reinstatement, what is unknown are factors that determine different magnitudes of reinstatement, a potential correlate to relapse vulnerability. In this thesis, we will investigate the contribution of glutamate and dopamine signaling in the nucleus accumbens core to reinstatement magnitude.

Glutamate neurotransmission is altered in animals withdrawn from repeated cocaine administration (Wolf et al., 2004, Kalivas et al., 2005), which likely contributes to addiction because abnormal activation of corticostriatal pathways correlates with craving in humans (Breiter et al., 1997, Volkow et al., 1999, Volkow et al., 2005) and is necessary for cocaine-seeking in rodents (Park et al., 2002, McFarland et al., 2003, Schmidt et al., 2005). Therefore, targeting glutamate neurotransmission therapeutically is likely to be an effective treatment for cocaine addiction.

Two distinct pools of extracellular glutamate in the nucleus accumbens core exist, synaptic and extrasynaptic. Synaptic glutamate derives primarily from glutamatergic neurons originating in the dorsomedial prefrontal cortex. Extrasynaptic glutamate is contributed by astrocytic vesicular (Hepp et al., 1999, Araque et al., 2000, Jourdain et al., 2007, Malarkey and Parpura, 2008) and non-vesicular sources (Baker et al., 2002, Moran et al., 2005). Non-vesicular glutamate provided primarily by system x_c^- regulates synaptic neurotransmission, and is decreased following withdrawal from cocaine self-administration (Baker et al., 2002, Moran et al., 2005, Moussawi et al., 2011b). Investigators have been able to directly target cystine-glutamate exchange to reduce cocaine-seeking (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008). In addition, restoration of cystine-glutamate exchange expression decreases cocaine-seeking

(Knackstedt et al., 2010), but little is known about multiple aspects during withdrawal; for example the contribution of diminished cystine-glutamate exchange to basal extracellular glutamate and cocaine-evoked glutamate in animals withdrawn from cocaine. In addition, the source of cocaine-induced decreases in cystine-glutamate has not been studied thoroughly.

The purpose of this thesis is to determine the contribution of diminished cystine-glutamate exchange in cocaine-seeking and what contributes to decreased cystine-glutamate following repeated cocaine. In order to address the former, we manipulate conditions that block cocaine-induced decreases in cystine-glutamate exchange. To address the latter, we will first determine neuronal signaling that regulates cystine-glutamate exchange. Below, I will describe in detail:

- Circuitry involved in cocaine-seeking: the motive circuit
- Behavioral models of addiction
- Cocaine-induced neuroplasticity relevant to cocaine-seeking
- The cellular and molecular basis of glutamate homeostasis
- Cystine-glutamate exchange by system x_c-

The motive circuit

In order to effectively identify pathological neuroplasticity in addiction, a detailed analysis of the circuitry contributing to addiction will be addressed. The motive circuit (figure i.1) is a combination of limbic circuit processing and motor control, and plays a central role in motivated behavior and addiction (Wise, 1984, Kalivas and McFarland, 2003, Kalivas et al., 2005, Schmidt et al., 2005). There exists two major divisions of the NAcc. The core division is the central part located mostly surrounding the anterior commissure. The shell division surrounds the core and occupies the ventral and medial portions of the NAcc. Though the core and shell share similar neuronal composition of medium spiny projection neurons (Meredith, 1999) in addition to cholinergic and parvalbumin interneurons (Kawaguchi et al., 1995), they differ in efferent and afferent pathways. Key among these distinct circuitry is the difference in excitatory input. For example, the nucleus accumbens core receives glutamatergic input primarily from the dorsomedial prefrontal cortex, whereas the nucleus accumbens shell receives input from

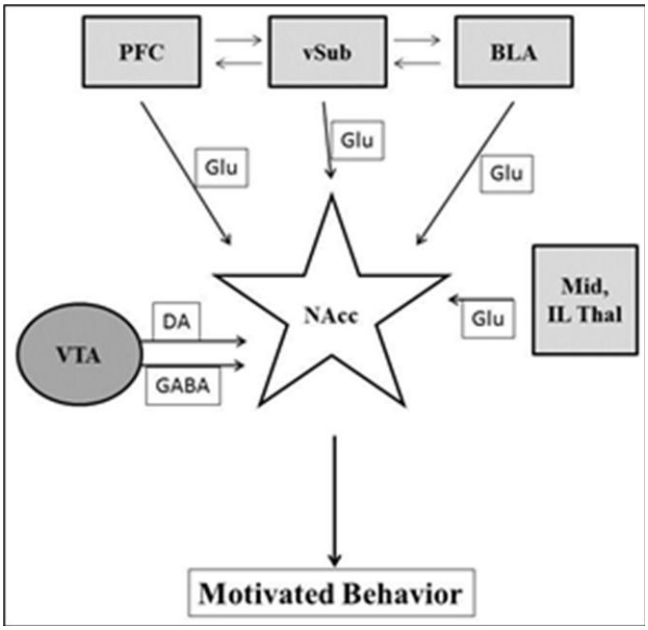


Figure i.1. Multiple projections to the nucleus accumbens interact to determine behavioral output. Glutamatergic projections from the PFC, vSub, BLA, and thalamus are excitatory glutamate projections. Dopaminergic projections from the VTA are modulatory. There also GABAergic inhibitory inputs from the VP and VTA. PFC, prefrontal cortex; vSub, ventral subiculum of the hippocampus; BLA, basolateral amygdala; Mid, IL, midline and intralaminar thalamaic nuclei; NAcc, nucleus accumbens; VP, ventral pallidum; VTA, ventral tegmental area.

the ventromedial prefrontal cortex, the ventral subiculum of the hippocampus, and the amygdala (Kelley and Domesick, 1982, Kelley et al., 1982, Groenewegen et al., 1987, Groenewegen et al., 1990, Kita and Kitai, 1990, McDonald, 1991). In addition to the differential input to the core and shell of the nucleus accumbens, they also differ in their functional downstream effects. While the nucleus accumbens core is the central behavioral output region for the motive circuit (McFarland and Kalivas, 2001, Kalivas and McFarland, 2003), the nucleus accumbens shell receives excitatory input to regulate dopamine cell activity in the ventral tegmental area (Floresco et al., 2001, Floresco et al., 2003, Goto and Grace, 2005). Investigators suggest that they also differ in their role in behavior and reward processing (Carlezon et al., 1995, Volkow and Fowler, 2000, Rodd-Henricks et al., 2002, Kalivas and McFarland, 2003, Robbins et al., 2008).

As portrayed in figure i.1, input to the nucleus accumbens can be divided into three major categories: excitatory (glutamatergic), inhibitory (GABAergic), and modulatory (dopamine and other neuromodulators). Excitatory input comes primarily from limbic areas such as the prefrontal cortex, entorhinal cortex, ventral subiculum of the hippocampus, and the basolateral amygdala (Kelley and Domesick, 1982, Kelley et al., 1982, Groenewegen et al., 1987, Kita and Kitai, 1990, McDonald, 1991, Berendse et al., 1992, Brog et al., 1993, Totterdell and Meredith, 1997, Reynolds and Zahm, 2005), as well as from the midline and intralaminar nuclei of the thalamus (Kelley and Stinus, 1984, Berendse and Groenewegen, 1990). Major inhibitory inputs derive from the ventral tegmental area and the ventral pallidum (Brog et al., 1993, Groenewegen et al., 1993, Churchill and Kalivas, 1994, Van Bockstaele and Pickel, 1995, Wu et al., 1996). Dopamine input comes mainly from the midbrain ventral tegmental area (Voorn et al.,

1986, Ikemoto, 2007). Understanding how these systems may be altered by chronic cocaine will be important to understanding the neural basis of addiction.

Models of addiction: Conditioned Place Preference

Conditioned place preference (CPP) is widely used to test the rewarding properties of drugs. It is based on classical conditioning whereby spatial cue-associated chambers are paired to an injection of either drug (cocaine) or saline. During the acquisition phase of CPP, an animal is first assessed for its "preference" for a particular chamber in a drug naïve state. This allows the experimenter to follow a biased, unbiased, or balanced procedure for the acquisition phase. In the biased method, the experimenter gives an injection of a drug when animals in a group enter the same chamber throughout the experiment, while the other is paired with a vehicle (i.e. saline) injection. The experimenter generally pairs the drug injection with the chamber the animals did not prefer in the drug naïve state. In the unbiased method, the drug paired compartment is picked at random between animals. In the balanced method, each compartment is assigned the same number of drug pairs across all animals. To determine the presence or absence of CPP, an experimenter would compare the time spent in drug- and vehicle-paired compartments (Parker and McDonald, 2000). Investigators appreciate this method based on the absence of contextual stimuli for which animals can acquire a secondary preference (i.e. light or noise) (Tzschentke, 2007). Whereas the development of place preference is a useful model for the rewarding properties of a drug, some experimenters prefer to utilize the extinction/reinstatement model of relapse.

During extinction, the animal will either received a saline injection regardless of the chamber entered, or will be allowed to fully explore both chambers without any

injections. Animals will continue to be placed in the chamber until a preference is extinguished. This stage in the paradigm allows an investigator to evaluate the motivational properties of the drug administered during the acquisition phase, in the absence of drug treatment.

Reinstatement of drug-paired behavior by a priming injection of the drug is a reliable model for drug craving and relapse (De Vries et al., 1998, Shalev et al., 2002). During the reinstatement phase of CPP, the animal receives an acute injection of drug or a stressor. Of particular interest is the ability of a variety of psychostimulants to cross-react and reinstate CPP. For example, animals extinguished from cocaine CPP exhibit reinstatement in the presence of a priming injection of methamphetamine, morphine, nicotine, and ethanol (Itzhak and Martin, 2002, Romieu et al., 2004). An acute stressor is also capable of reinstating CPP for cocaine (Lu et al., 2002, Sanchez et al., 2003).

There are advantages and disadvantages to the use of conditioned place preference as a model of addiction. Pharmacological treatment intended to reverse drug seeking during reinstatement may affect higher-level thought such as operant responding found in the self-administration model, which is less likely to alter cocaine-seeking in the CPP paradigm. In addition, the onset of "cocaine-seeking" by place preference is relatively quicker than the self-administration paradigm given that the animal is not required to learn operant responding. Disadvantages to this method include the lack of flexibility in the paradigm during particular phases of the experiment. For example, while the animal effectively prefers a chamber over the other, it is more difficult to determine magnitude of drug-seeking and its relation to compulsion.

Models of addiction: Self-administration

The self-administration model of addiction is a more widely used addiction paradigm. In this model, an animal is surgically implanted with an intravenous catheter and trained to respond (e.g. lever press or nose poke) to receive an infusion of drug (i.e. cocaine) during the acquisition/maintenance phase (Clark et al., 1961, Weeks, 1962). As in the acquisition phase of CPP, certain conditions of this phase allow the experimenter to measure rewarding and reinforcing properties of the drug, supporting the abuse potential of the drug (Johanson, 1990, Balster and Bigelow, 2003, Panlilio and Goldberg, 2007). The experimenter may manipulate the responding required to receive an infusion of drug, the two most common variations are fixed and progressive ratio [for review see (Arnold and Roberts, 1997)]. Fixed ratio schedules, when a fixed number of responses are required to receive drug infusion, are commonly used to measure the reinforcing properties of a drug. On the other hand, progressive ratio schedules, when the number of responses required to receive drug infusion "progressively" increases with each response, are commonly used to measure the effectiveness of the reinforce; sometimes considered a measure of the amount of work an animal is willing to exert, or willing to "pay", to receive the reinforcer. Some experimenters primarily utilize the acquisition/maintenance phase, this due to the fact that in the clinical setting a patient is not likely to remain abstinent unless under constant supervision.

Most investigators make use of the extinction/reinstatement model of self-administration. During extinction, drug-seeking is extinguished by replacing the infusion solution with saline or disconnection from the infusion pump. There are multiple variants of extinction/reinstatement such as between-session, within-session, and between-within-

session. In the between-session paradigm, the drug self-administration, extinction, and reinstatement occur on consecutive days; for example an animal gains drug-seeking on day one (or over multiple days), extinguishes drug-seeking the following day, and undergoes reinstatement the day after that. In the within-session paradigm drug self-administration, extinction, and reinstatement takes place sequentially within the same day. The between-within-session paradigm, by definition, involves drug self-administration across multiple days, and the extinction/reinstatement take place on a later day, but within the same day of each other. A common variant of the between-within paradigm involves a protracted withdrawal period following the self-administration phase (Fuchs et al., 2006), extinction across multiple days until responding within each session is minimal, and then reinstatement.

Following extinction, a stimulus such as drug, stress, or cue is provided to induce reinstatement of drug-seeking. Reinstatement of drug-seeking is defined as operant responding significantly higher than that observed on the last day of extinction. As in conditioned place preference, there exists cross sensitization of psychostimulants to induce reinstatement of drug seeking. Stress- (Erb et al., 1996, Shaham et al., 2000, Koob and Le Moal, 2001) and cue-induced (See, 2002) reinstatement are of interest due to their clinical relevance.

A distinct advantage of the self-administration paradigm is the ability to manipulate conditions to allow for a measure of compulsive behavior. Previous studies have shown that the level of cocaine intake during the maintenance phase of self-administration alters both the onset of compulsive drug seeking as measured by escalated intake during the maintenance phase, as well as elevated cocaine-induced reinstatement

after withdrawal (Ahmed and Koob, 1998), providing a preclinical basis for comparing recreational and addictive cocaine use.

Drug-induced plasticity in the nucleus accumbens and reinstatement

In the nucleus accumbens core, stimulation of glutamate (McFarland and Kalivas, 2001, Di Ciano et al., 2008) and dopamine (Spealman et al., 1999, Cornish and Kalivas, 2000, Park et al., 2002, Shalev et al., 2002) receptors is sufficient to produce drug-seeking. However, blockade of glutamate (Cornish et al., 1999, Cornish and Kalivas, 2000, McFarland et al., 2003) but not dopamine (McFarland and Kalivas, 2001, Anderson et al., 2003) receptors will abolish drug seeking. This suggests that long term changes in glutamate signaling in the nucleus accumbens core may underlie cocaine seeking behavior. This poses neuroplasticity involving glutamate in the nucleus accumbens core as a key mechanism that may be capable of producing compulsive drug seeking. In fact, studies have found long term changes in glutamate homeostasis and have linked these adaptations to cocaine-seeking (Pierce et al., 1996, Baker et al., 2003), as described below.

Glutamate homeostasis in the nucleus accumbens

There exists two distinct pools of extracellular glutamate, synaptic and extrasynaptic. As it pertains to the nucleus accumbens core, synaptic glutamate derives from glutamate terminals originating in the prefrontal cortex (Groenewegen et al., 1990, Berendse et al., 1992, Brog et al., 1993). Propagation of an action potential leads to an increase in calcium in the presynaptic terminal, resulting in docking and vesicular release of glutamate into the synaptic cleft.

Synaptic glutamate then targets both ionotropic and metabotropic glutamate receptors. There are two primary ionotropic receptors, a low affinity a-amino-3-hydroxy5-methyl-4-isoazole-proprionic acid (AMPA) and a high affinity N-methyl-D-aspartic acid (NMDA), both named for selective exogenous ligands capable of activating the receptors. The high affinity NMDA receptors are under constant blockage by a voltage sensitive Mg^{2+}-inhibitor. This magnesium blockade is removed when the cell is sufficiently depolarized, usually by activation of AMPA receptors. These conditions maintain a high signal/noise ration whereas sufficiently higher levels of glutamate are required to stimulate AMPA receptors, leading to relief of the Mg^{2+} blockade on NMDA receptors and further cellular depolarization.

Metabotropic glutamate receptors are G-protein coupled receptors categorized into three families based on sequence homology [for review, see (Nakanishi, 1994)]: Group I mGluRs, consisting of mGluR 1 and 5, activate G_q; Group II mGluRs, consistend of mGluR 2 and 3, activate G_i; and Group III mGluRs, consisting of mGluRs 4, 6, 7, and 8 activate Gi. Synaptic metabotropic glutamate receptors are primarily Group I. Group I receptors are primarily expressed on postsynaptic neurons and cholinergic neurons in the striatum (Kerner et al., 1997, Pisani et al., 2001a, Pisani et al., 2001b, Marino et al., 2002). Activation of postsynaptic Group I receptors facilitates synaptic signaling by contributing to LTP and LTD (Gubellini et al., 2003). Group II and III receptors have been localized to presynaptic excitatory terminals (Testa et al., 1998, Bradley et al., 1999, Kosinski et al., 1999). Stimulation of these receptors leads to inhibition of glutamatergic synaptic release in the striatum (Lovinger and McCool, 1995, Manzoni et al., 1997).

Synaptic glutamate is cleared from the synaptic space primarily by an excitatory amino acid transporter (EAAT), of which there are five (EAAT 1-5). Glutamate transport by an EAAT is coupled to the influx of three Na^+ molecules, one H^+ molecule, and efflux of one K^+ molecule (Zerangue and Kavanaugh, 1996, Levy et al., 1998, Owe et al., 2006). Given that glutamate is a negatively charged amino acid, the electrical gradient created by cotrans/antiport of these ions makes glutamate transport energetically favorable. EAATs are located both at the synaptic cleft and in the extrasynaptic space. This allows for functional separation of synaptic and extrasynaptic glutamate compartments.

As portrayed in figure i.2, extracellular glutamate derives from multiple sources and regulates synaptic activity. Glutamate release from astrocytes can occur through multiple mechanisms. Calcium-mediated glutamate release from astrocytes is dependent on storage of glutamate into vesicles and release by the SNARE complex (Parpura et al., 1995, Jeftinija et al., 1997, Hepp et al., 1999, Araque et al., 2000, Pasti et al., 2001, Montana et al., 2004, Crippa et al., 2006). Ca^{2+}-dependent glutamate regulates synaptic activity by targeting

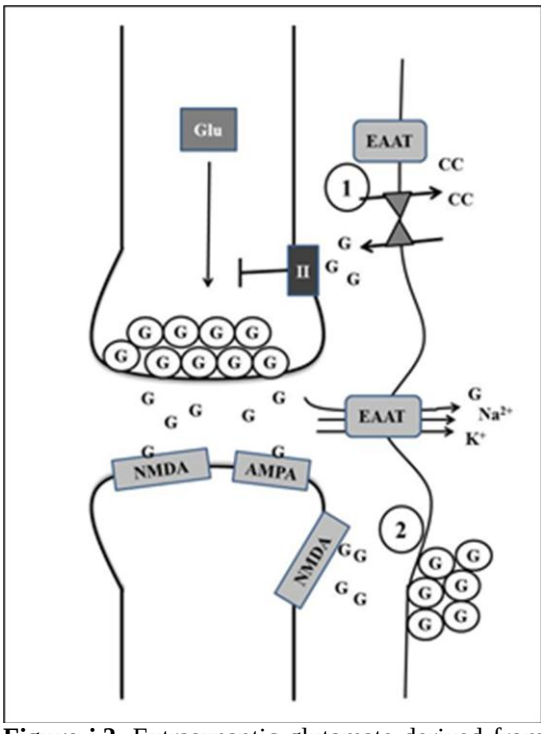


Figure i.2. Extrasynaptic glutamate derived from astrocytes can regulate synaptic activity. (**1**) Nonvesicular glutamate from cystine-glutamate exchange stimulates presynaptic inhibitory Group II receptors, decreases synaptic release of neurotransmitter. (**2**) Ca^{2+}-dependent vesicular release of glutamate activates extrasynaptic NMDA receptors, potentiating synaptic activity. CC, cystine; G, glutamate; II, group II metabotropic glutamate receptors; NMDA, NMDA receptor; AMPA, AMPA receptor; EAAT, excitatory amino acid transporter.

extrasynaptic NMDA receptors (D'Ascenzo et al., 2007, Jourdain et al., 2007). Other astrocytic sources of extrasynaptic glutamate include hemichannels (Ye et al., 2003), volume-sensitive organic ion channels (Strange et al., 1996, Basarsky et al., 1999, Ye et al., 2003), and cystine-glutamate exchange (Bannai, 1986, Murphy et al., 1990, Warr et al., 1999, Baker et al., 2002, Moran et al., 2005).

Cystine-glutamate exchange by system x_c-

Cystine-glutamate exchange by system x_c- contributes to extrasynaptic glutamate by stoichiometric exchange of an extracellular cystine molecule for an intracellular glutamate molecule (Bannai, 1986, Murphy et al., 1990, Warr et al., 1999, Baker et al., 2002, Moran et al., 2005). System x_c- is comprised of two protein subunits. The heavy chain subunit, 4f2hc is nonspecific for amino acid transporter. The functional subunit, xCT is specific for system x_c- and is necessary for cystine-glutamate exchange (Shih and Murphy, 2001). Cystine transport by cystine-glutamate exchange is chloride-dependent and sodium-independent

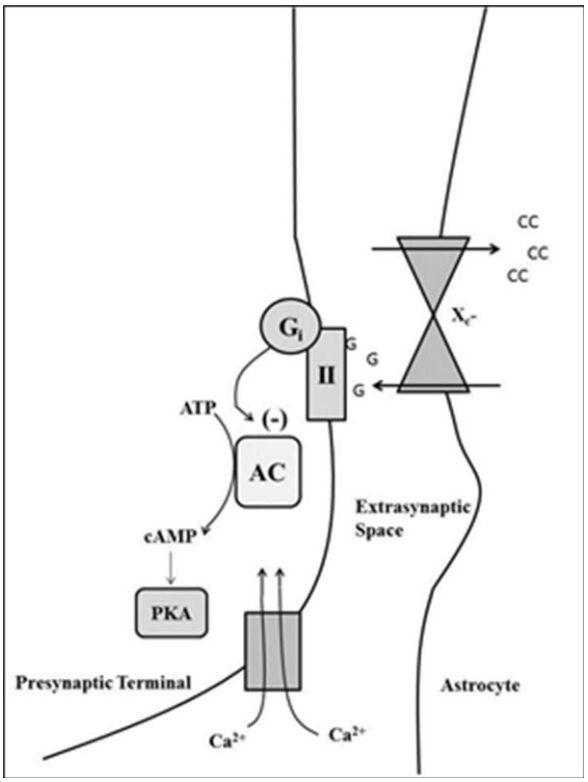


Figure i.3. Cystine-glutamate exchange regulates synaptic activity. Glutamate derived from cystine-glutamate exchange stimulates presynaptic Group II receptors, which activates G_i signaling. G_i reduces adenylyl cyclase activity and eventually PKA activity, which under normal circumstances increases activity of Ca^{2+}channels that are necessary for synaptic release of neurotransmitter. CC, cystine; G, glutamate; II, group II metabotropic glutamate receptors (mGluR2/3); AC, adenylyl cyclase, PKA, protein kinase A.

(Waniewski and Martin, 1984, Bannai, 1986), a key property that distinguishes it from other more common amino acid transporters such as EAATs. Figure i.3 illustrates how extrasynaptic glutamate from cystine-glutamate exchange stimulates presynaptic inhibitory group II metabotropic glutamate receptors (mGluR2/3) (Baker et al., 2002, Moran et al., 2005, Moussawi et al., 2011b), rendering it an essential mechanism for normal synaptic function. Cocaine-induced decreases in cystine-glutamate exchange have been found in animals withdrawn from chronic cocaine self-administration (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Moussawi et al., 2011b). As well, withdrawal from chronic cocaine results in a decrease in basal extracellular glutamate (Pierce et al., 1996, Baker et al., 2003), though others have found this not to be the case (Reid and Berger, 1996). Decreases in basal extracellular glutamate likely contribute to the augmented cocaine-evoked glutamate in the nucleus accumbens of cocaine-withdrawn animals as extrasynaptic nonvesicular glutamate regulates synaptic transmission (Baker et al., 2002, Moran et al., 2005, Moussawi et al., 2011b). To overcome this deficit, we have targeted cystine-glutamate exchange both during reinstatement. Stimulating cystine-glutamate exchange can reduce cocaine-primed reinstatement after protracted withdrawal.

Given that altered system x_c^- activity is essential to elevated drug seeking, it is important to know why it is diminished. Cystine-glutamate exchange has been shown regulated in a tonic manner by interleukin-1β (Jackman et al., 2010) and fibroblast growth factor 2 (FGF-2) (Liu et al., 2011), and acutely by Group II receptors (Xi et al., 2002a), protein kinase A (Baker et al., 2003) and protein kinase C (Tang and Kalivas, 2003). Further, evidence exists suggesting that dopamine regulates GLT-1 expression in

the striatum (Brito et al., 2009). In addition, a novel neuropeptide, pituitary adenylyl cyclase activating polypeptide (PACAP) has been shown to regulate glutamate homeostasis by regulating activity of glial glutamate transporters in the striatum (Figiel and Engele, 2000). In this dissertation, we will investigate regulation of cystine-glutamate exchange by dopamine and PACAP.

Pituitary adenylyl cyclase activating polypeptide (PACAP)

PACAP was originally discovered and cloned from ovine hypothalamus in 1989, and so named for its ability to stimulate adenylate cyclase and cAMP formation. Its first discovery in the rodent brain was in the anterior pituitary from the hypothalamus (Miyata et al., 1989). The first peptide discovered was 38 amino acids long (PACAP38), followed by discovery and cloning of PACAP27, comprising of the first 27 amino acids of PACAP38 (Miyata et al., 1990).

As illustrated in figure i.4, PACAP is likely co-released with glutamate from glutamatergic neurons. This is based on studies showing that PACAP colocalizes with vesicular glutamate transporter 2 (VGLUT2) (Engelund et al., 2010). This concept is highly significant given that

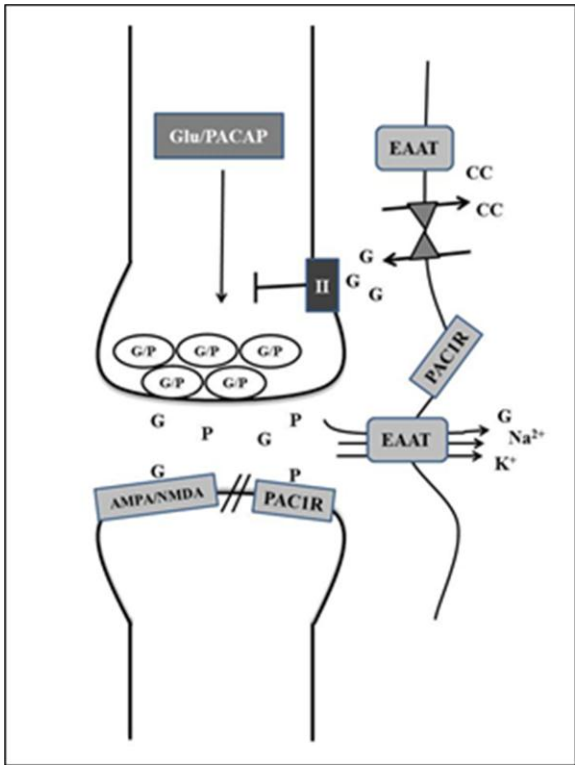


Figure i.4. PACAP is likely co-released with glutamate. An action potential leads to the Ca^{2+}-dependent release of PACAP from the nerve terminal. PACAP stimulates postsynaptic and astrocytic PAC1R in the nucleus accumbens core. At this point, not much is known on how PACAP is removed from the extracellular space or how it is metabolized.

glutamatergic signaling plays a major role in cocaine seeking. Unfortunately, not much more is known regarding the regulation of PACAP release and how it is affected following repeated consumption of cocaine.

PACAP binds to three G-protein coupled receptors, PAC1, VPAC1, and VPAC2. PAC1 shows a higher affinity for PACAP over vasoactive intestinal peptide (VIP), whereas VPAC1 and VPAC2 have similar affinities to the two neuromodulators. PAC1 receptor signaling is linked to both Gs or Gq mediated G-protein signaling. Three different isoforms of PAC1 exist, which help determine which G-protein signaling mechanism is activated. PAC1null and PAC1hop, of which there are hop1 or hop2, can activate both Gq and Gs signaling thereby either increasing adenylyl cyclase activity and increasing intracellular cAMP by Gs, as well as increasing intracellular calcium through Gq mediated events. On the other hand, the hip isoform is only capable of increasing adenylyl cyclase activity through Gs (Holighaus et al., 2011). There also exists a PAC1hiphop isoform that is not well known. While sparse, literature suggests that PAC1null and PAC1hop are the most abundant isoforms in the adult rat brain (Grimaldi and Cavallaro, 1999, Lutz et al., 2006, Mustafa et al., 2007).

PACAP is distributed extensively throughout the central nervous system, including the hypothalamus, cerebral cortex, amygdala, nucleus accumbens, hippocampus, and cerebellum (Arimura et al., 1991, Hannibal, 2002); as well as the autonomic nervous system (Sundler et al., 1996). The distribution of PACAP-projecting neurons has been studied using *in situ* hybridization of PACAP mRNA as well as with intracellular immunohistochemical labeling of PACAP. The highest density of PACAP-expressing fibers and cells bodes are found in the hypothalamus, particularly the

paraventricular nucleus, the supraoptic nucleus (Koves et al., 1991, Hannibal et al., 1995a, Hannibal et al., 1995b), the arcuate nucleus (Hannibal et al., 1995b, Murase et al., 1995, Das et al., 2007), and the ventromedial hypothalamic nucleus (Hannibal et al., 1995a, Skoglosa et al., 1999). Further, there is evidence of PACAP mRNA as well as immunoreactive fibers in cortical regions, including the frontal cortex, neocortex, cingulate, and entorhinal cortex (Mikkelsen et al., 1994, Hannibal et al., 1995a). In the hippocampus, there is a moderate density of immunoreactive fibers and cell bodies containing PACAP in the CA1, CA2, CA3, and dentate gyrus (Koves et al., 1991, Skoglosa et al., 1999, Hannibal, 2002).

The use of PACAP and PAC1R knockout mice has revealed some phenotypes in behavioral output. For example, investigators have shown that PACAP knockout mice exhibit elevation in locomotion in the presence of anxiolytic behavior (Hashimoto et al., 2001). More relevant to this purpose of this dissertation, one study has shown a lack of difference in methamphetamine-induced behavioral sensitization when comparing wild-type and PACAP-deficient mice (Fujii et al., 2007); in short, though both groups exhibited behavioral sensitization, neither group exhibited a significantly difference level of sensitization.

Summary

The current state of cocaine addiction research posits cystine-glutamate exchange as a viable target for treatment (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008). Table 1 lists the current state of cocaine-seeking as it pertains to diminished cystine-glutamate exchange. Animals receiving sufficiently high levels of cocaine during self-administration exhibit an escalation in consumption during self-administration (Ahmed and Koob, 1998, Ahmed et al., 2003) and a decrease in cystine-glutamate exchange (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008).

Table 1. Known and unknown contributions of diminished cystine-glutamate exchange to cocaine-seeking.

Known

- Cystine-glutamate exchange regulates neurotransmission by stimulating presynaptic inhibitor Group II receptors
- Repeated cocaine by self-administration leads to a decrease in cystine-glutamate exchange by system x_c-
- Animals with cocaine-induced decreases in cystine-glutamate exchange also exhibit diminished basal extracellular glutamate in the nucleus accumbens
- Animals withdrawn from repeated cocaine, and thus diminished cystine-glutamate exchange, exhibit cocaine-evoked glutamate after withdrawal
- Acute restoration of cystine-glutamate exchange reduces cocaine-seeking

Unknown

- Does diminished cystine-glutamate exchange a consequence of repeated cocaine, does it contribute to basal extracellular glutamate, cocaine-evoked glutamate, and reinstatement
- What contributes to the magnitude of cocaine seeking (i.e. reinstatement)
- What contributes to decreased cystine-glutamate exchange in cocaine-withdrawn animals
- What regulates cystine-glutamate exchange

The aim of this dissertation will be to further advance the field by revealing mechanisms that contribute to relapse vulnerability. As shown in Table 2, we will further characterize neuroplasticity contributing to compulsive drug seeking and relapse vulnerability. As well, we attempt to distinguish differential neurotransmitter signaling in animals exhibiting elevated reinstatement. Though much is known regarding regulation of glutamate homeostasis through glutamate transporters, little is known regarding the regulation of cystine-glutamate exchange. We attempt to determine neuronal signaling that may regulate system x_c- and how it is effected following withdrawal from cocaine self-administration. Altogether, findings in this dissertation should further the field of cocaine addiction research, and reveal potential novel therapeutic targets.

Table 2. Aims and experimental approaches in this dissertation.

AIM	**EXPERIMENT**
Determine the contribution of cocaine induced diminished cystine-glutamate exchange to basal extracellular glutamate, cocaine-evoked glutamate, and reinstatement.	Block cocaine-induced neuroplasticity by repeated stimulation of cystine-glutamate exchange during the maintenance phase of self-administration
Determine what contributes to the magnitude of reinstatement to help understand relapse vulnerability	Alter cocaine intake during the maintenance phase of self-administration and determine differences in cocaine-evoked neurochemistry, neuronal signaling, and cystine-glutamate exchange
Determine the source of diminished cystine-glutamate exchange in cocaine-withdrawn animals	Measure regulation of cystine-glutamate exchange by neuronal signaling, then determine if this is altered in cocaine-withdrawn animals

CHAPTER 1

Contribution of diminished system x_c^- to cocaine-evoked neurochemistry and cocaine-seeking

Introduction

Repeated cocaine alters glutamate neurotransmission even following protracted withdrawal (Wolf et al., 2004, Kalivas et al., 2005), and this likely contributes to addiction since abnormal activation of corticostriatal pathways correlates with craving in humans (Breiter et al., 1997, Volkow et al., 1999, Volkow et al., 2005) and is necessary for cocaine seeking in rodents (Park et al., 2002, McFarland et al., 2003, Schmidt et al., 2005). Revealing cellular mechanisms underlying altered corticostriatal activation should advance our understanding of the neurobiological basis of addiction and identify novel therapeutic targets (Volkow and Fowler, 2000, Dackis, 2004).

Models of pathological glutamate signaling proposed to underlie addiction need to account for the existence of multiple pools of extracellular glutamate. Aside from synaptic glutamate maintained by vesicular release, extrasynaptic glutamate is sustained primarily by nonvesicular release (Herrera-Marschitz et al., 1996, Timmerman and Westerink, 1997). In support, basal extrasynaptic glutamate sampled using microdialysis are largely independent of vesicular glutamate (Westerink, 1995). Glutamate transporters may partition the two pools by limiting glutamate overflow from the synapse into extrasynaptic compartments (Danbolt, 2001), and restricting entry of nonvesicular glutamate into synapses (Jabaudon et al., 1999). Although confined to the extrasynaptic compartment, nonvesicular glutamate regulates neurotransmission by stimulating group II metabotropic glutamate receptors (mGluRs) (Baker et al., 2002, Xi et al., 2002a) which are extrasynaptic receptors capable of inhibiting vesicular release (Baskys and Malenka, 1991, Cochilla and Alford, 1998, Schoepp, 2001). Thus, extrasynaptic receptors permit

crosstalk between the two pools and indicate that altered nonvesicular glutamate release may contribute to pathological glutamate signaling linked to addiction.

Cystine-glutamate exchange via system x_c^- may be critical in the capacity of extrasynaptic glutamate to regulate corticostriatal signaling in the normal and pathological states. First, nonvesicular release from cystine-glutamate exchange maintains basal extracellular glutamate in the nucleus accumbens (Baker et al., 2002, Xi et al., 2002a), and thereby regulates the extent of endogenous group II mGluR stimulation (Baker et al., 2002, Melendez et al., 2005, Moran et al., 2005). Repeated cocaine blunts system x_c^- activity which leads to reduced basal and likely increased cocaine-evoked glutamate in the nucleus accumbens that persists for at least three weeks after the last cocaine treatment (Baker et al., 2003). These changes are relevant for drug seeking since N-acetylcysteine, a cysteine prodrug used to drive system x_c^- (Williamson and Meister, 1981, Meister, 1985, Pileblad and Magnusson, 1992), blocks cocaine-evoked glutamate in the nucleus accumbens and subsequent cocaine-induced reinstatement (Baker et al., 2003). What is lacking is a clear demonstration that the changes in glutamate and cocaine-seeking require reduced cystine-glutamate exchange.

In these studies, we test the hypothesis that diminished system x_c^- activity is part of the neuroplasticity contributing to compulsive drug seeking. As shown in the introduction, stimulating cystine-glutamate exchange during self-administration prevents escalation of cocaine-seeking. We hypothesize that preventing escalation of cocaine-seeking alters cocaine-induced neuroplasticity associated with reinstatement after protracted withdrawal. Further, we also hypothesize that escalation of cocaine-seeking results in long-term changes in cystine-glutamate exchange.

Materials and Methods

Animals

These experiments utilized male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275–325 grams upon arrival. Rats were individually housed in a temperature-controlled colony room with a 12-h reversed light/dark cycle. Housing conditions and experimental protocols were approved by the Marquette University Institutional Animal Care and Use Committee and carried out according to the NIH Guide for the Care and Use of Laboratory Animals (revised 1996).

Surgeries

Rats were implanted with indwelling catheters under ketamine HCl (100 mg/kg, IP, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (2 mg/kg, IP, Lloyd Laboratories, Shenandoah, IA, USA) anesthesia. A silicon tubing catheter (Dow Corning Co., Midland, MI; 0.64 mm ID; 1.19 mm OD) was implanted such that it entered the jugular vein through the right posterior facial vein and terminated at the right atrium. The catheter was sutured to the vein at the entry point. The distal aspect of the catheter, which consisted of a 22-gauge guide cannula (Plastics One Inc., Roanoke, VA) attached with dental acrylic to a piece of polypropylene monofilament surgical mesh (Atrium Medical, Co., Hudson, NH), exited 2 cm posterior to the scapulae. Throughout the experiment, catheters were filled daily with a heparin solution (83 i.u./ml; Elkins-Sinn, Inc., Cherry Hill, NJ) and capped when disconnected from the leash/delivery line assembly. Rats were also implanted with indwelling bilateral guide cannulae (20 gauge, 14 mm; Plastics One, Roanoke VA) using the following coordinates derived from Paxinos and Watson (1986): + 0.9 mm anterior, ± 2.5 mm mediolateral to Bregma, and −4.4 mm from the surface of

the skull at a 6° angle from vertical. The placement of the active region of the microdialysis probe, which began 1 mm beyond the ventral tip of the guide cannulae, was primarily in the NAcc although regions immediately dorsal and ventral to this were also likely sampled. Following surgery, rats were given at least five days to recover prior to testing. During this time, rats were provided acetaminophen (480 mg/L) in their drinking water and injected daily with a sterile cefazolin antibiotic solution (15 mg, IV; West-Ward Pharmaceutical Co., Eatontown, NJ).

Cocaine Self-Administration Training

Self-administration occurred in operant conditioning chambers (ENV-008CT, MED-Associates Inc., St Albans, VT, USA) housed in sound attenuating cubicles (ENV-016M, MED-Associates Inc.) and equipped with two retractable levers, two stimulus lights, and a water bottle. At least five days after surgery, rats were food restricted with water available ad libitum; food restriction continued for the duration of the experiment (e.g., through reinstatement testing) and involved daily administration of 15 grams of rat chow in the late afternoon or immediately following an operant session. Rats were then placed into the operant conditioning chambers overnight and responses on the lever designated as active resulted in the delivery of food pellets under a fixed ratio 1 schedule of reinforcement. Daily food training continued until subjects received at least 150 food rewards in a session, which typically occurred following the first session. *During the acquisition phase of the experiment*, all rats underwent drug self-administration training during daily 2-hr sessions in which operant responses on the active lever were reinforced with an infusion of cocaine (0.5 mg/kg/200µl infusion, IV, National Institute on Drug Abuse, Bethesda, MD, USA) under a fixed ratio 1 schedule of reinforcement. Each

reinforced lever response resulted in the illumination of the stimulus light located above the active lever and was followed by a 25-s time-out period. Responding on a second, inactive lever located on the back wall was recorded but had no programmed consequences. Acquisition of cocaine self-administration was operationally defined as < 10% variation in daily responding over at least three consecutive sessions. *During the maintenance phase of the experiment*, rats were assigned to self-administer cocaine under low- (0.5 mg/kg/200µl infusion, IV, 2 hrs/day) or high-intake conditions (1.0 mg/kg/200µl, IV, 6 hrs/day) for 11 days.

Extinction/Reinstatement testing

After completing eleven maintenance self-administration sessions, rats remained in their home cages for seven days prior to extinction training. The seven day delay was used to ensure an adequate drug-free period prior to reinstatement, even in rats that quickly extinguished responding. Extinction training involved placing rats into the operant conditioning chambers for 2-hr/day as described above except each active lever press now resulted in an infusion of saline. This continued until the mean number of lever presses was ≤ 10 responses across at least three sessions, at which point rats were tested for drug-primed reinstatement. The reinstatement test day was identical to each extinction session except rats were injected with cocaine (10 mg/kg, IP). Because the mean number of extinction sessions needed to meet criteria (± SEM) was 12.2 ± 1.2, reinstatement testing occurred approximately 19 days after the last self-administration session.

In vivo Microdialysis Testing

On the night before testing, microdialysis probes, constructed as previously described (Baker et al., 2003), were inserted into indwelling guide cannula. Rats were

then housed overnight in the self-administration chambers. The next day, dialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.15% phosphate buffer saline, pH 7.4) was pumped through the probes at a rate of 1 μl/min for at least 3 hr to permit an adequate period of time for neurotransmitter levels to stabilize. Afterwards, twenty min samples were collected as described below for each experiment.

Quantification of Glutamate

The concentration of glutamate in dialysis samples was quantified by comparing peak heights from samples and external standards using HPLC coupled to fluorescence detection. Precolumn derivatization of glutamate with opthaldehyde was performed using a Shimadzu LC10AD VP autosampler. The mobile phase consisted of 13% acetonitrile, 100 mM Na_2HPO_4, and 0.1 mM EDTA, pH 5.90. Glutamate was separated using a reversed-phase column (4 μm; 140 × 6.0 mm; Phenomenex, Torrance, CA), and detected using a Shimadzu 10RF-AXL fluorescence detector with excitation and emission wavelengths of 320 and 400 nm, respectively.

Quantification of Dopamine

Dopamine concentrations were quantified by comparing peak heights from samples and external standards using HPLC coupled to electrochemical detection. The mobile phase consisted of 15% acetonitrile, 10% methanol, 150 mM NaH2PO4, 4.76 mM citric acid, 3 mM SDS, and 50 μM EDTA, pH 5.6. Dopamine was separated using a reversed-phase column (3 μm; 80 × 3.2 mm; ESA Inc., Bedford, MA) and detected using an ESA Coulochem II detector coupled to two electrodes set at −0.075 V and +0.25 V, respectively.

[^{35}S] Cystine uptake ex vivo

Rats were decapitated and the brains were rapidly extracted and cut into 2 mm coronal slices using a brain matrix. Tissue punches (1.25 mm diameter; Stoelting, Wood Dale, IL) were collected from the nucleus accumbens core and incubated at 37°C for ~30 min on a nylon bolting cloth platform, submerged beneath 2 mm of standard buffer (Lobner and Lipton, 1993); the standard buffer contained 124 mM NaCl, 3.0 mM KCl, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 4 mM glucose, equilibrated with 95% O_2, 5% CO_2, pH 7.4. After a 30 min wash, the tissue was incubated for 30 min in standard buffer containing [^{35}S]cystine (4 μCi/ml; GE Healthcare, Piscataway, NJ). D-Aspartate (1 mM) was also added to the incubation buffer to prevent uptake via X_{AG}. After 30 min incubation, the tissue punches were washed four times in ice-cold standard buffer and dissolved in 250 μl of 1% SDS. One aliquot (100 μl) was used to measure [^{35}S]cystine uptake by scintillation counting; a second aliquot (100 μl) was used to assess protein content by the BCA method. Additional punches from each subject were processed as described above with the exception that unlabeled CPG [(*S*)-4-carboxyphenylglycine] (1 mM) was added 10 min before and for the duration of the 30 min incubation with [^{35}S]cystine (4 μCi/ml). These values reflect labeling independent of system x_c^- and were subtracted from the total labeling obtained from each subject to get a measure that solely reflected uptake through cystine–glutamate exchange.

Experiment 1: Impact of repeated N-acetylcysteine on cystine-glutamate exchange in cocaine-withdrawn animals

N-acetylcysteine (Sigma, St. Louis, MO) is a cysteine prodrug that stimulates cystine-glutamate exchange (Moldeus et al., 1981, Meister, 1985). Animals underwent

self-administration training and *during the maintenance phase* were pretreated with saline or N-acetylcysteine (60mg/kg, IP) one hour prior to daily saline or cocaine self-administration (1.0mg/kg/200μL infusion, IV 6 hrs/day) for 11 days. This produced four treatment groups, Sal-Sal, NAC-Sal, Sal-Coc, and NAC-Coc. After protracted withdrawal of at least 7 days, animals were rapidly decapitated and measure for *ex vivo* cystine uptake in the nucleus accumbens core as described above.

Experiment 2: Impact of repeated N-acetylcysteine on basal extracellular glutamate

Animals underwent self-administration training and *during the maintenance phase* were pretreated with saline or N-acetylcysteine (60mg/kg, IP) one hour prior to daily saline or cocaine self-administration (0.5mg/kg/200μL infusion, IV 2hrs/day) for 11 days. This produced four treatment groups, Sal-Sal, NAC-Sal, Sal-Coc, and NAC-Coc. Animals then underwent 7 days of withdrawal and extinction training. After the last day of extinction training, animals were placed into the self-administration chambers and underwent microdialysis as described above. To determine the level of basal extracellular glutamate, the first 3 samples were quantified for glutamate and area under the curve (AUC) was calculated.

Experiment 3: Impact of repeated N-acetylcysteine on cocaine-evoked glutamate

Animals underwent self-administration training and *during the maintenance phase* were pretreated with saline or N-acetylcysteine (60mg/kg, IP) one hour prior to daily saline or cocaine self-administration (0.5mg/kg/200μL infusion, IV 2hrs/day) for 11 days. This produced four treatment groups, Sal-Sal, NAC-Sal, Sal-Coc, and NAC-Coc. Animals then underwent 7 days of withdrawal and extinction training. After the last day of extinction training, animals were placed into the self-administration chambers and

underwent microdialysis as described above. To determine the magnitude of cocaine-evoked glutamate, after the baseline was established *all animals* received an injection of cocaine (10mg/kg, IP) and the self-administration chamber was activated. Glutamate levels were normalized to the mean of the 3 baseline samples.

Experiment 4: Impact of repeated N-acetylcysteine on cocaine-primed reinstatement

Animals underwent self-administration training and *during the maintenance phase* were pretreated with saline or N-acetylcysteine (60mg/kg, IP) one hour prior to daily saline or cocaine self-administration (0.5mg/kg/200μL infusion, IV 2hrs/day) for 11 days. This produced four treatment groups, Sal-Sal, NAC-Sal, Sal-Coc, and NAC-Coc. Animals then underwent 7 days of withdrawal and extinction training. After the last day of extinction training, animals were placed into the self-administration chambers and underwent reinstatement as described above.

Histology

Rats included in the microdialysis studies were given an overdose of pentobarbital (60 mg/kg, IP), and the brains were fixed by intracardiac infusion of 0.9% saline followed by 2.5% formalin solution. Brains were removed and stored in 2.5 % formalin for at least seven days prior to sectioning. The tissue was then blocked and coronal sections (100 μM) were cut and stained with cresyl violet to verify probe placements.

Statistical analyses

SPSS software (version 16) was used to perform the statistical analyses. Data were analyzed using analysis of variance (ANOVA) with self-administration access conditions and N-acetylcysteine dose as potential between-subject factors and time (20-min interval) as a repeated factor. *Post hoc* comparisons were conducted using t-tests when only two

data points were compared. Other *post hoc* comparisons are as noted. Note, the *p* value required for significance in the post hoc tests are adjusted downward by the total number of comparisons conducted to control for alpha inflation.

Results

Experiment 1: Impact of repeated N-acetylcysteine on cystine-glutamate exchange in cocaine-withdrawn animals

Figure 1.1 illustrates the impact of daily N-acetylcysteine pretreatment on cocaine-induced plasticity involving system x_c-. A comparison of [^{35}S]cystine transport produced an interaction between N-acetylcysteine and cocaine treatment conditions ($F_{1,31}$= 7.53; p=0.01). Post hoc analyses revealed that rats pretreated with saline before cocaine self-administration exhibited reduced [^{35}S]cystine transport relative to saline controls (Dunnett's t, $p<0.05$). Furthermore, rats pretreated with N-acetylcysteine before cocaine self-administration failed to exhibit diminished cystine–glutamate exchange, although cystine uptake was measured 23–34 d after the last treatment of either cocaine or N-acetylcysteine (Dunnett's t, $p<0.05$).

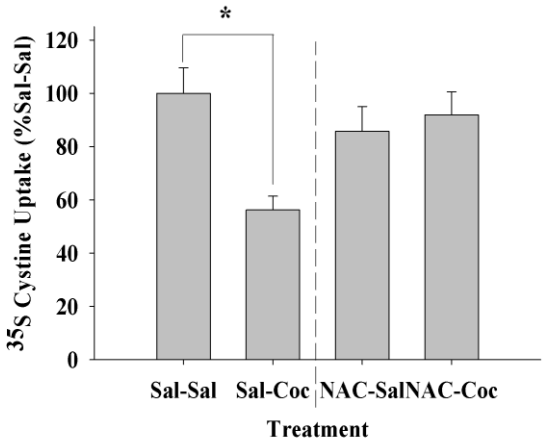


Figure 1.1. Daily N-acetylcysteine prevents long-term deficits in cystine-glutamate exchange in the nucleus accumbens produced by repeated cocaine. Cystine transport was measured in tissue punches from the nucleus accumbens after withdrawal from self-administration. *Significant difference from Sal-Sal controls.

Experiment 2: Impact of repeated N-acetylcysteine on basal extracellular glutamate

Cystine-glutamate exchange is diminished in the nucleus accumbens of animals withdrawn from cocaine self-administration (Baker et al., 2003). As well, cystine-glutamate exchange regulates synaptic neurotransmission (Baker et al., 2002, Moran et al., 2005, Moussawi et al., 2011b). We wanted to determine if chronic stimulation of

cystine-glutamate exchange and therefore constant regulation of synaptic signaling, during cocaine self-administration will reverse deficits found in basal and cocaine-evoked glutamate. Figure 1.2 illustrates that chronic treatment with the cysteine prodrug N-acetylcysteine restores basal glutamate during withdrawal. A comparison of basal glutamate (before cocaine primer) as measured by area under the curve yielded a significant interaction between N-acetylcysteine and cocaine treatments (ANOVA: $F_{1,37}$=4.05, p<0.05). *Post hoc* analyses revealed that a reduction in basal glutamate relative to saline controls was found in rats pretreated with daily saline before cocaine self-administration sessions (Dunnet's t, p<0.05), a significant difference was also found when comparing with rats that had been treated with N-acetylcysteine during self-administration training (Dunnet's t, p<0.05).

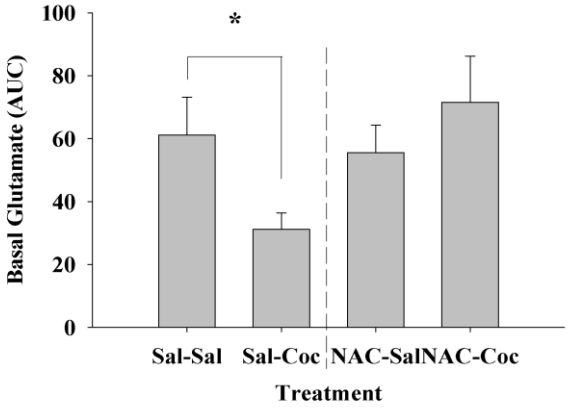


Figure 1.2. Daily N-acetylcysteine prevents long-term changes in extracellular basal glutamate levels in the nucleus accumbens produced by repeated cocaine. Basal glutamate levels are depicted as area under the curve calculated using samples collected before the cocaine injection. *Significant difference from Sal-Sal controls.

Experiment 3: Impact of repeated N-acetylcysteine on cocaine-evoked glutamate

Increased glutamate signaling in the nucleus accumbens is necessary for cocaine-seeking during reinstatement. As well, others have shown that only animals withdrawn from repeated cocaine exhibit cocaine-evoked glutamate. We wanted to determine if cocaine-evoked (10 mg/kg, IP) glutamate is absent in animals that underwent self-administration but exhibit normal levels of cystine-glutamate exchange and basal

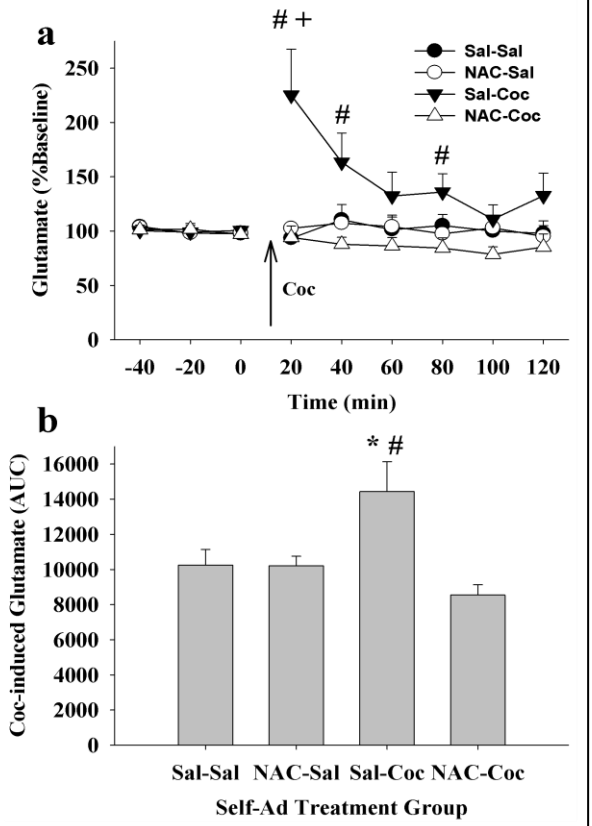


Figure 1.3. Daily N-acetylcysteine prevents changes in cocaine-evoked extracellular glutamate levels in the nucleus accumbens produced by repeated cocaine. Cocaine-evoked glutamate is presented as percent change from baseline (**a**) or area under the curve calculated using samples collected after the cocaine injection (**b**). *Significant difference from Sal-Sal controls; +Significant difference from the last baseline sample; #Significant difference from NAC-Coc rats.

glutamate. An ANOVA comparing cocaine-evoked glutamate during reinstatement (Figure 1.3) with N-acetylcysteine and cocaine treatment as between-subjects measures and time (20 min samples) as a repeated measure yielded a significant three-way interaction ($F_{8,296}$=2.05, $p<0.05$). *Post hoc* analyses revealed that animals pretreated with saline prior to daily cocaine self-administration session exhibited significantly higher levels of cocaine-evoked glutamate during reinstatement (Figure 1.3a; Dunnet's t, $p<0.05$), though there was no significant difference found in animals treated with N-acetylcysteine prior to daily cocaine self-administration. When measuring area under the curve (AUC), a significant interaction between N-acetylcysteine and cocaine treatment was observed (Figure 1.3b; $F_{1,37}$=4.05, $p<0.05$). *Post hoc* analyses yielded a significant increase in glutamate in rats pretreated with saline before cocaine self-administration when compared to animals pretreated with N-acetylcysteine (Dunnet's *t*, $p<0.05$).

Experiment 4: Impact of repeated N-acetylcysteine on cocaine-primed reinstatement

The above findings show that diminished cystine-glutamate exchange contributes to altered basal and cocaine-evoked glutamate. We wanted to determine if it also contributes to cocaine-primed reinstatement. Figure 1.4 illustrates the impact of repeated N-acetylcysteine on the last three extinction sessions and cocaine-primed (10mg/kg, IP) reinstatement in animals withdrawn from short-access cocaine-self administration conditions (0.5mg/kg/200μL infusion, IV, 2 hrs/day). N-acetylcysteine administered during self-administration did not alter the number of extinction session required to meet criteria when compared to cocaine animals pretreated with saline (Mean ± SEM: NAC, 11 ± 2; Sal, 13.6 ± 1.7; Student's *t*, p>0.05). In contrast a comparison of lever pressing during the reinstatement test day and the last three extinction sessions produced a three-way interaction between test day, N-acetylcysteine treatment, and cocaine treatment (ANOVA: $F_{3,111}$=3.06, p<0.05). *Post hoc* analyses revealed that a cocaine primer reinstated extinguished drug seeking, evident as a significant increase in lever pressing on the reinstatement test *relative to the last extinction session*, but only in rats that had been treated with saline and cocaine during self-administration (Dunnet's *t*, p<0.05); this group of rats also exhibited significantly higher levels of responding on the reinstatement test

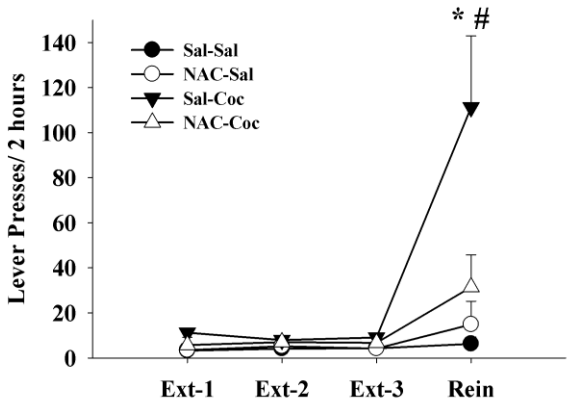


Figure 1.4. Daily N-acetylcysteine prevents cocaine-induced reinstatement. Data depict mean ± SEM behavior during the last three extinction sessions and following a cocaine-primer (10mg/kg, IP) for groups treated as designated during self-administration. *Significant difference from the last extinction session; Dunnet's *t*, p<0.05. #Significant difference from all other groups; Dunnet's *t*, p<0.05.

day compared to all other groups (Dunnet's *t*, $p<0.05$). In contrast, cocaine rats pretreated with N-acetylcysteine failed to reinstate cocaine-seeking despite testing 2-3 weeks after the last N-acetylcysteine treatment.

The above findings suggest that *diminished cystine-glutamate exchange* by cocaine-self-administration contributes to the following:

- **Diminished basal extracellular glutamate in the nucleus accumbens**
- **Cocaine-evoked glutamate in the nucleus accumbens**
- **Cocaine-primed reinstatement**

These findings reveal diminished cystine-glutamate exchange by system x_c- as a necessary event for decreased basal glutamate, increased cocaine-evoked glutamate, and cocaine-primed reinstatement. Thus reduced system x_c- activity may be critical to the transition of drug-seeking from casual to compulsive. To further test this, we examined the status of cystine-glutamate exchange under conditions that have been shown to produce elevated levels of cocaine intake and heightened cocaine-primed reinstatement. Previous studies have suggested that animals consuming elevated levels of cocaine during self-administration exhibit elevated reinstatement (Ahmed and Koob, 1998, Ahmed et al., 2003). In the next section, we test the hypothesis that high-intake rats (1.0mg/kg/200μL infusion, IV, 6 hrs/day) exhibiting heightened reinstatement of cocaine-seeking display a more robust cocaine-induced increase in extracellular glutamate in the nucleus accumbens compared to low-intake rats (0.5mg/kg/200μL infusion, IV, 2 hrs/day) given that glutamate is necessary for cocaine-seeking (Park et al., 2002, McFarland et al., 2003, Schmidt et al., 2005). Given that diminished cystine-

glutamate exchange is essential to cocaine-evoked neurotransmission and reinstatement, as shown in the previous section, we hypothesize that animals receiving high-intake cocaine during self-administration exhibit diminished cystine-glutamate exchange compared to low-intake animals.

Cocaine-evoked extracellular glutamate is not affected by cocaine self-administration intake conditions

Glutamate neurotransmission in the nucleus accumbens following cocaine self-administration is altered and is necessary for cocaine-seeking as shown above and in previous findings (Pierce et al., 1996, Torregrossa et al., 2008, Suto et al., 2009). We hypothesized that animals consuming higher levels of cocaine show augmented glutamate neurotransmission during reinstatement. In animals that received either high- or low-intake conditions, we measured cocaine-evoked glutamate by microdialysis during the reinstatement phase. Figure 1.5a illustrates that there was no significant difference in cocaine-evoked glutamate release between low- and high-intake animals. An ANOVA with time (20 minute samples) as a repeated measure and intake conditions as a between subjects factor yieled a significant main effect of time ($F_{10,350}$=4.32, p<0.001), no significant main effect of intake ($F_{1,35}$=0.101, p=0.752), nor an interaction between the variables ($F_{10,350}$=0.773, p=0.655). *Post hoc* analyses revealed significant increases in cocaine-evoked glutamate for both low- and high-intake animals in 4 of the samples collected after the cocaine injection regardless of intake condition (Fisher LSD, p<0.008). To verify the lack of a difference in cocaine-evoked glutamate between low- and high-intake animals, we measured area under the curve (AUC) for samples collected after the

cocaine injection. Figure 1.5b illustrates no significant difference in cocaine-evoked glutamate as measured by AUC (t_{35}=0.5, p=0.691). Figure 1.5c illustrates that low-intake animals exhibited lower cocaine-primed reinstatement when compared to high intake animals despite no difference in extracellular glutamate (t_{33}=4.476, p<0.001).

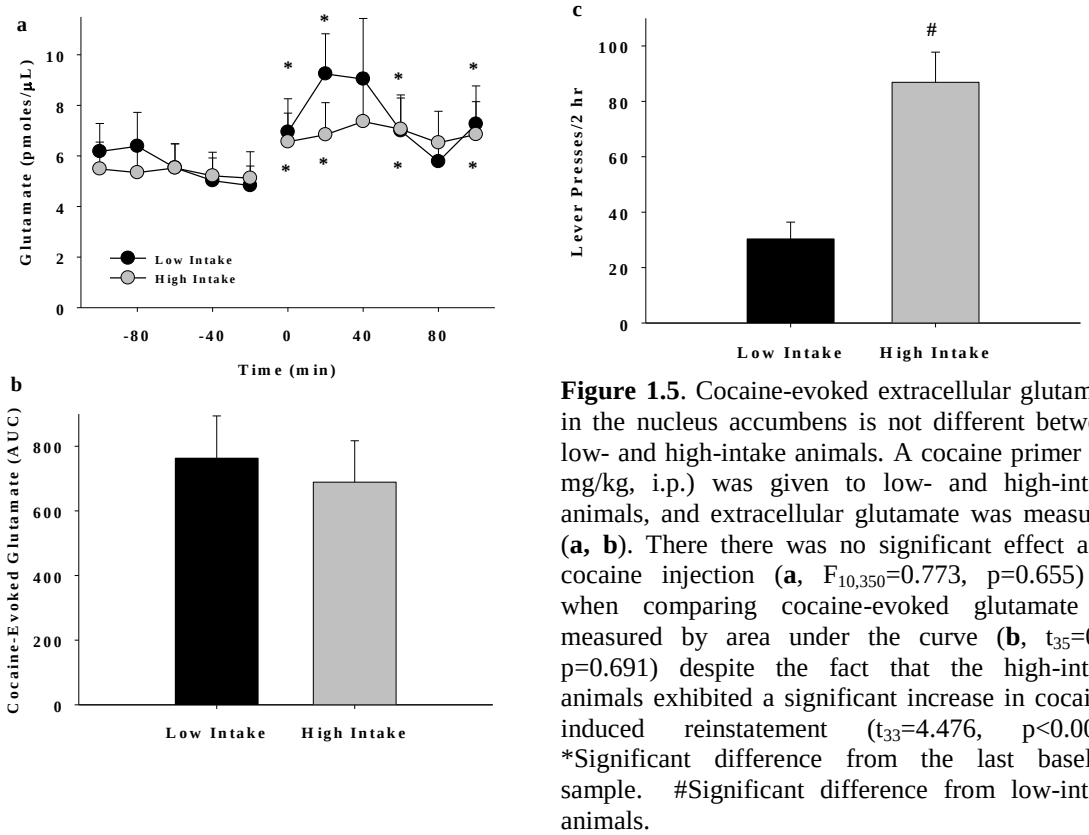


Figure 1.5. Cocaine-evoked extracellular glutamate in the nucleus accumbens is not different between low- and high-intake animals. A cocaine primer (10 mg/kg, i.p.) was given to low- and high-intake animals, and extracellular glutamate was measured (**a, b**). There there was no significant effect after cocaine injection (**a**, $F_{10,350}$=0.773, p=0.655) or when comparing cocaine-evoked glutamate as measured by area under the curve (**b**, t_{35}=0.5, p=0.691) despite the fact that the high-intake animals exhibited a significant increase in cocaine-induced reinstatement (t_{33}=4.476, p<0.001). *Significant difference from the last baseline sample. #Significant difference from low-intake animals.

High-intake cocaine self-administration conditions augment cocaine-evoked extracellular dopamine

Glutamate is necessary for cocaine-seeking and cocaine-evoked glutamate is exhibited only in animals withdrawn from repeated cocaine. However, glutamate is not essential for heightened reinstatement as shown above. Dopamine is not necessary for cocaine-primed reinstatement as by blocking dopamine signaling in the nucleus

accumbens does not abolish reinstatement. Conversely, elevating dopamine signaling in the nucleus accumbens either by microinjection of dopamine or activating dopamine receptors is sufficient to induce reinstatement. Therefore, we hypothesized that while dopamine is not necessary for reinstatement, it determines the magnitude of reinstatement. Figure 1.6a illustrates extracellular dopamine levels in the NAcc before and after a cocaine injection (10 mg/kg, i.p.) in high- and low-intake rats when tested after withdrawal and extinction from cocaine self-administration. An ANOVA with intake conditions as a between-subjects factor and time (20 min samples) as a repeated measure yielded a significant interaction ($F_{10,230}$=4.14, p<0.001). An augmented cocaine-induced increase in extracellular dopamine was evident in high-intake rats when areas under the curve were calculated using all of the postinjection samples and compared across low- and high-intake conditions (Fig 1.6b; t_{23}=2.143, p=0.043). As expected, the high-intake group in this microdialysis study exhibited augmented reinstatement relative to low-intake rats (Fig. 1.6c; t_{32}=5.689, p<0.001).

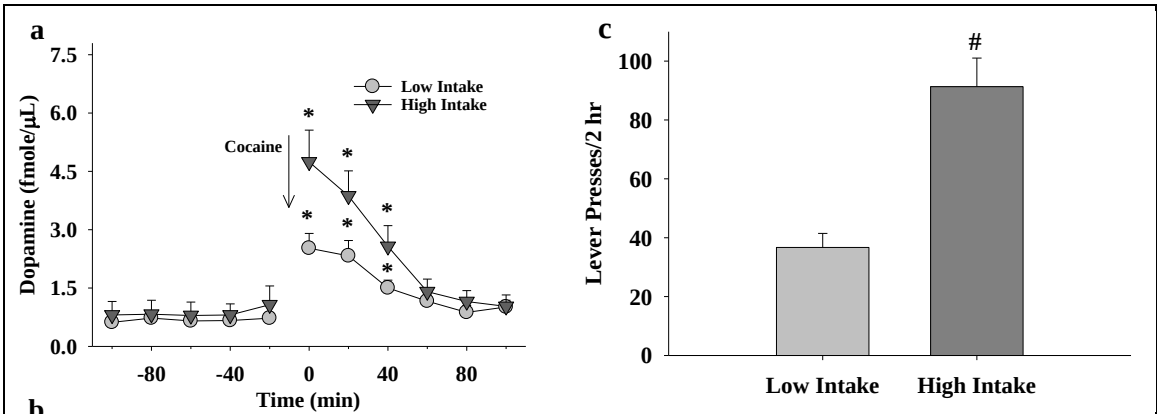


Figure 1.6. High-intake cocaine self-administration augments both cocaine-evoked extracellular dopamine levels in the nucleus accumbens core presented as femtomoles/μL as well as reinstatement. A cocaine primer (10 mg/kg, i.p.) was given to low- and high-intake animals, and extracellular dopamine was measured (**a, b**). *Post hoc* analyses reveal a significant effect of intake condition as measured by areas under the curve (**b**, t_{23}=2.143, p=0.043), as well as reinstatement (**c**, t_{32}=5.689, p<0.001). *Significant difference from last baseline sample (Fisher LSD, p<0.05). #Significant difference from low-intake animals.

Impact of D1 receptor blockade on cocaine-induced reinstatement by low- and high-intake rats

As shown above, cocaine-evoked dopamine levels in the nucleus accumbens are altered based on cocaine intake conditions. We want to determine if this effect is behaviorally relevant. We hypothesized that elevated dopamine stimulates D1-like dopamine receptors leading to augmented reinstatement. Therefore, we reverse dialyzed the D1-like dopamine receptor antagonist, SCH-23390 (0 or 10μM) and determined if receptor blockade alters cocaine-primed reinstatement. This concentration of SCH-23390 was determine based on previous published usage (Anderson et al., 2003, Schmidt et al., 2006, Schmidt and Pierce, 2006). Figure 1.7 depicts responding during extinction and

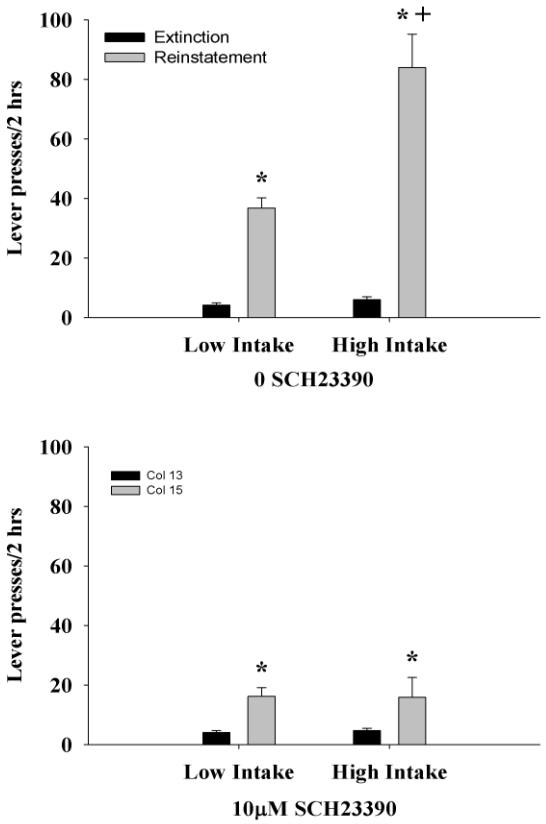


Figure 1.7. Augmented cocaine-primed reinstatement in high-intake animals is due to stimulation of D1-like dopamine receptoirs. Data depict responding during extinction and cocaine-primed reinstatement testing in the absence (***a***) or presence (***b***) of SCH-23390. SCH-23390 (0 and 10 μM) was infused into the nucleus accumbens core via reverse dialysis beginning 1 hour before the cocaine primer (10mg/kg, IP) and for the duration of the experiment. $^{+}$Significant difference from low-intake rats (*t* test, $p < 0.001$). *Significant difference from extinction responding (***a***, *t* test, $p < 0.001$; ***b***, ANOVA main effect, $p < 0.05$).

reinstatement testing in rats when conducted in the absence (Fig. 1.7a) or presence of SCH-23390 (Fig. 1.7b) infused into the NAcc via reverse dialysis. An ANOVA with intake conditions and SCH-23390 concentration (0 or 10μM) as between-subjects factors and experimental test phase (extinction, reinstatement) as a repeated measure yielded a three-way interaction ($F_{1,31}$=16.99, p<0.001). Two separate ANOVAs (in the presence or absence of SCH-23390 infusion) were then conducted to deconstruct the three-way interaction with intake conditions as a between-subjects variable and experimental phase as a repeated measure. In the absence of SCH-23390, a two-way interaction ($F_{1,17}$=25.93, p<0.001) was obtained. Subsequent post hoc comparisons indicated that both intake groups exhibited significant reinstatement, and that the high-intake group exhibited higher levels of responding during the test for reinstatement relative to low-intake rats (t test, $p < 0.0125$). In the presence of SCH-23390, there was a main effect of experimental phase ($F_{1,14}$=14.28, p<0.05), without

a significant main effect of intake conditions ($F_{1,14}$=0.002, p=0.965) or an interaction between the variables ($F_{1,14}$=0.028, p=0.870).

N-Acetylcysteine blocks augmented cocaine-evoked dopamine in high-intake cocaine self-administration animals

Restoring or preventing reduced cystine-glutamate exchange by system x_c- in cocaine-withdrawn animals lowers reinstatement and eliminates cocaine-evoked glutamate after withdrawal. We wanted to determine if augmented cocaine-evoked dopamine found in high-intake animals during reinstatement is affected by cystine-glutamate exchange. We pretreated high-intake animals with the cysteine prodrug N-acetylcysteine just prior to a cocaine primer and measured extracellular dopamine. Figure 1.8a illustrates the effectiveness of N-acetylcysteine (NAC) in reducing cocaine-evoked extracellular dopamine in high-intake cocaine self-administration animals. An ANOVA

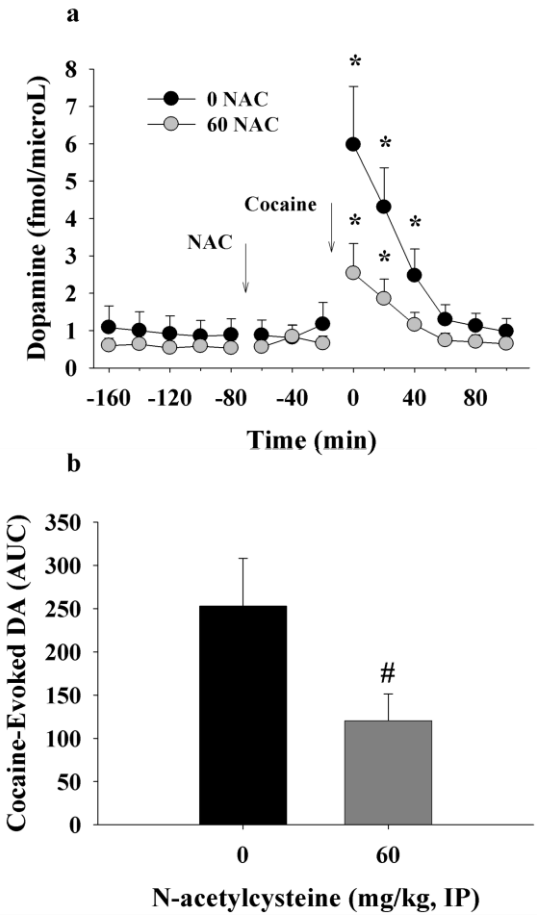


Figure 1.8. Stimulating cystine-glutamate exchange diminishes elevated cocaine-evoked dopamine in animals withdrawn from high-intake cocaine self-administration. Extracellular dopamine in the nucleus accumbens core presented as femtomoles/mL (**a**) or area under the curve (**b**) calculated from the samples collected following the cocaine injection. Microdialysis was used to sample extracellular dopamine in the nucleus accumbens core before and after injection of N-acetylcysteine (NAC, 0 or 60 mg/kg, i.p., N = 10-11/group) and cocaine (10 mg/kg, i.p.). *Significant difference from baseline. #Significant difference from 0 N-acetylcysteine group.

with NAC (0 or 60 mg/kg i.p.) as a between-subjects variable and time (20 min samples) as a repeated measure yielded a significant interaction ($F_{13,247}$=14.9850, p<0.001). Pretreatment with NAC diminished cocaine-induced increases in extracellular dopamine when areas under the curve were calculated using all of the post-injection samples and compared across pretreatment (Fig 1.8b;

t_{19}=2.148, p=0.045).

When combining all animals from figure 1.5 and figure 1.6, we found a significant correlation between reinstatement and cocaine-evoked dopamine as measured by areas under the curve (Pearson Correlation; r^2 = 0.313, p<0.05), suggesting a link between the cocaine-evoked neurochemical and behavioral responses.

Self-administration intake conditions result in differential altered system x_c- activity

Figure 1.9 illustrates that animals withdrawn from high-intake cocaine self-administration exhibit diminished cystine-glutamate exchange in the nucleus accumbens core when compared to low-intake animals as measured by [^{14}C]cystine transport *ex vivo* (t_7=2.36, p<0.05).

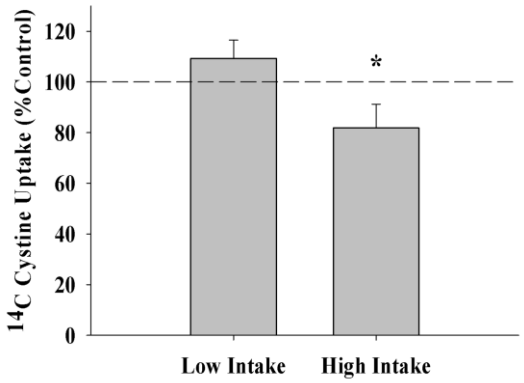


Figure 1.9. High-intake conditions decrease cystine-glutamate exchange. *Ex vivo* tissue punches from the nucleus accumbens core are measured for [^{14}C]cystine transport and normalized to drug naïve animals following withdrawal from cocaine self-administration. *Significant difference from low-intake animals (t_7=2.36, p<0.05).

Discussion

The aim of this chapter was to show that diminished cystine-glutamate exchange contributes to cocaine-seeking behavior. Stimulating cystine-glutamate exchange with the cysteine prodrug N-acetylcysteine (NAC) just prior to daily cocaine self-administration blocked cocaine-induced deficits in cysteine-glutamate exchange. Animals with functional cystine-glutamate exchange exhibited normal basal extracellular glutamate and a lack of cocaine-evoked extracellular glutamate in the nucleus accumbens during reinstatement. These findings suggest that diminished cystine-glutamate exchange by system x_c- and basal extracellular glutamate are essential to cocaine-evoked glutamate and reinstatement, likely due to the fact that extracellular glutamate from cystine-glutamate exchange regulates neurotransmission (Baker et al., 2002, Moran et al., 2005, Moussawi et al., 2011b).

Though we were able to block cocaine-induced deficits in cystine-glutamate exchange by repeated treatment with N-acetylcysteine during the maintenance phase of self-administration, it is unclear how this takes place. We show that drug naïve animals do not exhibit cocaine-evoked glutamate in the nucleus accumbens (figure 1.2), however others have shown that an acute intraperitoneal injection leads to a significant increase in glutamate (Smith et al., 1995). Therefore, it is possible that repeated stimulation of cystine-glutamate exchange reduces levels of cocaine-evoked glutamate from the first day of self-administration. It is unlikely that cystine-glutamate exchange stimulation prevents cocaine-induced neuroplasticity by its actions at dopamine terminals. This is based on previous findings that suggest blocking D2 (Caine et al., 2002) or D1 dopamine

(Koob et al., 1987) receptors during self-administration either is ineffective or exacerbates cocaine-seeking during the maintenance phase.

Cocaine-induced neuroplasticity may occur in the absence of diminished cystine-glutamate exchange. By chronic stimulation of cystine-glutamate exchange during the maintenance phase of self-administration we deduce that preventing diminished cystine-glutamate exchange was the primary contributor to blocking cocaine-induced neuroplasticity in the form of cocaine-evoked glutamate, diminished basal glutamate, and reinstatement behavior. Most data supports that cystine-glutamate exchange regulates synaptic glutamate (Baker et al., 2003, Moran et al., 2003, Moussawi et al., 2011a) and dopamine as shown in this chapter. However, cocaine can alter levels of other neurotransmitters [for review see (Torregrossa and Kalivas, 2008)]. Therefore, it is possible that diminished cystine-glutamate exchange may be coincidental in cocaine-induced neuroplasticity. To address this, a future experiment may involve blocking cystine-glutamate exchange during reinstatement in animals that received repeated N-acetylcysteine. If diminished cystine-glutamate exchange is central to cocaine-evoked glutamate and reinstatement, CPG should restore levels of reinstatement to what is observed in rats not receiving NAC during self-administration.

An alternate mechanism that may contribute to altered glutamate signaling in the nucleus accumbens core is glutamate transport. As mentioned previously, glutamate transporters regulate the levels of glutamate both in the extrasynaptic pool and at the synaptic cleft (Danbolt, 2001). Interestingly, glutamate transporters are highly coupled to cystine-glutamate exchange. For example, GLT-1 and system x_c- can be expressed by the same transcription factor, Nrf2 (Shih et al., 2003), and pharmacological agents, such as

the β-lactam antibiotic ceftriaxone, that restores expression of cystine-glutamate exchange also restores expression of GLT-1 (Knackstedt et al., 2010). Diminished glutamate transport by GLT-1 would also contribute to elevated cocaine-evoked glutamate. In order to address this, an experimenter may need to determine if repeated N-acetylcysteine prevents cocaine-induced decreased GLT-1 expression.

We are particularly interested in neuroplasticity contributing to relapse vulnerability. We hypothesized that animals exhibiting elevated cocaine-primed reinstatement due to high-intake cocaine during the maintenance phase of self-administration also exhibit elevated cocaine-evoked glutamate neurotransmission during reinstatement. This approach was used because it has been previously shown that the magnitude of behavioral output during reinstatement correlates with the amount of cocaine intake during self-administration (Sutton et al., 2000), which is consistent with findings presented in this chapter. Surprisingly, animals consuming elevated levels of cocaine showed no changes in cocaine-evoked glutamate during reinstatement when compared to low-intake animals, despite an increase in cocaine-primed reinstatement.

Increasing levels of dopamine specifically in the nucleus accumbens core is sufficient to induce reinstatement (Spealman et al., 1999, Cornish and Kalivas, 2000, Park et al., 2002, Shalev et al., 2002). However, blocking dopamine receptors does not abolish cocaine-primed reinstatement (McFarland and Kalivas, 2001, Anderson et al., 2003). Given this, we did not expect to find differences in cocaine-evoked dopamine in animals withdrawn from low- versus high-intake self-administration conditions. Interestingly, the cocaine-evoked extracellular dopamine response in the nucleus accumbens was elevated in high- compared to low-intake animals. More importantly, we

were able to reduce this augmented dopamine response by pretreatment with N-acetylcysteine, suggesting that diminished cystine-glutamate exchange may play a role in augmented dopamine release and reinstatement in high-intake animals. Animals withdrawn from high-intake cocaine self-administration exhibited diminished cystine-glutamate exchange compared to low-intake animals. These findings suggest that decreased system x_c- activity may contribute to augmented dopamine and reinstatement in animals with heightened relapse susceptibility.

N-acetylcysteine blunts cocaine-induced reinstatement

Stimulating cystine-glutamate exchange with the cysteine prodrug N-acetylcysteine (NAC) reduces cocaine-primed reinstatement as shown in this chapter and previously (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008). Further, increased glutamate neurotransmission is necessary for cocaine craving in humans (Breiter et al., 1997, Volkow et al., 2005) and essential for reinstatement of cocaine-seeking in rodents (Park et al., 2002, McFarland et al., 2003, Schmidt et al., 2005), therefore it may seem counterintuitive that stimulating glutamate release through cystine-glutamate exchange is effective in reducing cocaine-seeking as seen in this chapter. However, it is important to be mindful of the purpose of multiple pools of extracellular glutamate. System x_c- releases glutamate into the extrasynaptic compartment, activating presynaptic group II metabotropic glutamate receptors (Baker et al., 2002, Moran et al., 2005), then reducing the release of synaptic vesicular glutamate (Moran et al., 2003, Moran et al., 2005, Moussawi et al., 2011b) and dopamine as shown in this chapter.

Contribution of system x_c- and dopamine in the NAcc to heightened relapse vulnerability

Animals that received high levels of cocaine during self-administration (1.0 mg/kg/200μL infusion, IV, 6 h/d) showed elevated levels of cocaine-evoked dopamine when compared to animals that received lower levels (0.5 mg/kg/200μL infusion, IV, 2 h/d), contrary to findings by others (Ahmed et al., 2003, Lack et al., 2008). However, in the mentioned studies, animals underwent microdialysis within 24 hours of the final self-administration session and in chambers that were not associated with cocaine self-administration. This is important as it has been shown previously that drug-paired cues contribute to cocaine-evoked dopamine (Ito et al., 2000). Therefore, it is likely that microdialysis taken place in the self-administration chambers, such as described in this chapter, provides a more accurate assessment of cocaine-evoked dopamine. The addiction field would benefit by investigating the contribution of drug-associated cues to cocaine-evoked drug seeking and dopamine. Interestingly, previous findings have shown that inhibiting the ventral subiculum of the hippocampus can block cocaine-primed reinstatement (Sun and Rebec, 2003). This is significant because the hippocampus is highly associated with spatial cues (Baylis and Moore, 1994, 1996), making it a likely region contributing to the differential release of dopamine in cocaine-withdrawn rats.

Animals withdrawn from high-intake conditions exhibited diminished cystine-glutamate exchange compared to low-intake animals. Further, when stimulating cystine-glutamate exchange in high-intake animals just prior to cocaine-primed reinstatement, the augmented extracellular dopamine response was diminished. This suggests that diminished system x_c- activity contributes to augmented dopamine signaling in the nucleus accumbens, as illustrated in figure 1.10, leading to elevated cocaine-seeking.

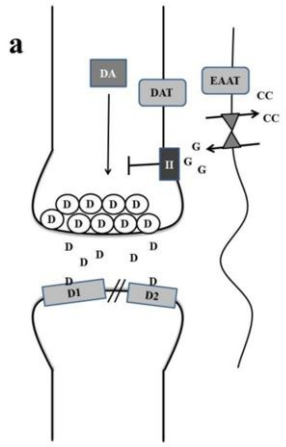
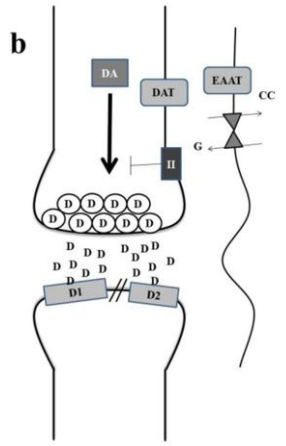
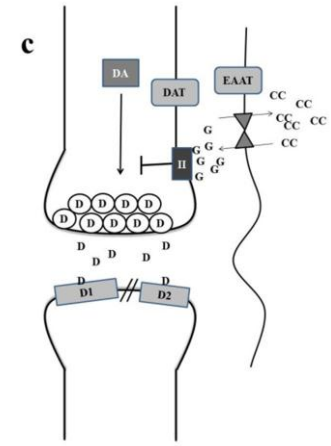
	Cocaine-evoked dopamine in High-intake animals	Cocaine-evoked dopamine in High-intake animals pretreated with NAC
		

Figure 1.10. Representative schematic depicting changes in cystine-glutamate exchange, dopamine neurotransmission, and how treatment with N-acetylcysteine reduces augmented dopamine release. (**a**) Under normal drug naïve conditions, dopamine release is tightly regulated by cystine-glutamate exchange and group II receptors. (**b**) Animals withdrawn from cocaine self-administration have diminished cystine-glutamate exchange and therefore less tone on group II receptors, disinhibiting synaptic release of dopamine. (**c**) Treating cocaine-withdrawn animals with N-acetylcysteine increases extrasynaptic glutamate and tone on group II receptors, returning dopamine release to almost normal levels.

One possible link between augmented reinstatement and elevated cocaine-evoked dopamine are the receptor affinities for dopamine. For example, D2-like dopamine receptors, which are considered inhibitory because they are G_i-linked, have a higher affinity for dopamine. This means that lower levels of dopamine stimulate primarily D2-like dopamine receptors. Conversely, D1-like dopamine receptors are considered excitatory because they are G_s-linked, and have a lower affinity for dopamine. Therefore, it is likely that augmented extracellular dopamine is required for activation of D1-like dopamine receptors. This is in line with findings that inactivation of D1-like or D2-like receptors in the core individually can block cocaine-primed reinstatement [for review see (Anderson et al., 2003, Schmidt et al., 2005, Schmidt et al., 2006)].

By manipulating conditions that altered reinstatement behavior and cocaine-induced plasticity, we were able to support the predictive validity of self-administration as a preclinical model for addictive behavior. The findings in this chapter suggest that high levels of drug intake results in the emergence of drug-induced adaptations that contribute to heightened relapse vulnerability. High-intake rats exhibit a more robust increase in extracellular levels of nucleus accumbens dopamine, but not glutamate, following a cocaine injection and this likely contributes to augmented reinstatement. The results are surprising because increased extracellular glutamate, but not dopamine, in the nucleus accumbens is necessary for cocaine-primed reinstatement (Cornish et al., 1999, Cornish and Kalivas, 2000, Park et al., 2002, Anderson et al., 2003, McFarland et al., 2003, Bachtell et al., 2005, Schmidt et al., 2005). In addition, diminished cystine-glutamate exchange was exhibited in high-intake animals when compared to low-intake animals. These findings also suggest that dysregulation of system x_c- in high-intake animals may be due to altered dopamine signaling. We will investigate the regulation of cystine-glutamate exchange by dopamine receptors. But first, in chapter 2 we establish a model for measuring regulation of system x_c-.

CHAPTER 2

Development of assays for measurement of cystine-glutamate exchange

Introduction

Cystine-glutamate exchange by system x_c^- is an important regulator of synaptic activity that is altered in neuronal disorders such as schizophrenia (Baker et al., 2008, Lavoie et al., 2008) and cocaine addiction (Baker et al., 2003, Moran et al., 2005, Amen et al., 2011). In order to determine how cystine-glutamate exchange is altered in diseased states, we must first investigate how it is regulated. The aim of this chapter is to establish *in vitro* and *in vivo* assays that allow us to efficiently measure regulation of system x_c^-. Cystine-glutamate exchange stoichiometrically exchanges extracellular cystine for an intracellular glutamate at a 1:1 ratio. We will compare the use of glutamate release and cystine uptake as a reliable measure of cystine-glutamate exchange. We will also look to determine the cell type from which cystine-glutamate exchange takes place *in vitro*. In order to determine if we can regulate cystine-glutamate exchange *in vitro,* we will attempt to modulate cystine transport by cAMP-dependent protein kinase (PKA). Cystine-glutamate exchange contributes to neuronal dopamine signaling in the nucleus accumbens core. In order to use mixed cultures from the cortex, we attempt to show that there is similar regulation of neuronal signaling in the cortex as is found in the accumbens.

Others have used glutamate transport to measure cystine-glutamate exchange activity (Knackstedt et al., 2010); the advantage to this method is the availability and stability of [^{3}H]glutamate when compared to cystine. This requires that the experimenter distinguish between Na^+-dependent and Na^+-independent uptake. While cystine is primarily transported by system x_c^- (Murphy et al., 1989), glutamate can be transported both through system x_c^- and system X_{AG}. In order to distinguish system x_c^- from system

X_{AG} transport, the experimenter must utilize Na^+-free media, an unacceptable condition when attempting to regulate cystine-glutamate exchange through receptor stimulation and kinase activity. This chapter will show that the use of [^{14}C]cystine transport *in vitro* is a preferable model for measuring cystine-glutamate exchange activity.

System x_c- is composed of two proteins, the nonspecific 4f2hc heavy chain subunit, and the functional xCT subunit. xCT has consensus phosphorylation sites and has previously been shown regulated by PKA and PKC (Baker et al., 2003, Tang and Kalivas, 2003). In this chapter, we support these findings using *in vitro* methods utilized in chapters 3 and 4.

Cystine-glutamate exchange regulates synaptic activity *in vivo* (Baker et al., 2002) and in slice preparation from the nucleus accumbens (Moran et al., 2005, Moussawi et al., 2011b). *In vitro* studies in this dissertation are performed in cortical cultures. In this chapter, we present data suggesting cystine-glutamate exchange regulates neurotransmission in the cortex as well, supporting the use of cortical cultures in future studies.

Materials and Methods

Animals

Animal housing conditions and experimental protocols were approved by the Marquette University Institutional Animal Care and Use Committee and carried out according to the NIH Guide for the Care and Use of Laboratory Animals (revised 1996).

Mouse cortical cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15–16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Astrocyte enriched glial cultures were prepared as described for mixed cultures except they are from cortical tissue taken from post-natal day 1–3 mice (Choi et al., 1987, Schwartz and Wilson, 1992). Neuronal cultures were prepared as described for mixed cultures with cytosine arabinoside (10 μM) added to the cultures 48 h after plating to inhibit glial replication. Less than 1% of cells in these cultures stain for glial fibrillary acidic protein (GFAP) (Dugan et al., 1995). Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

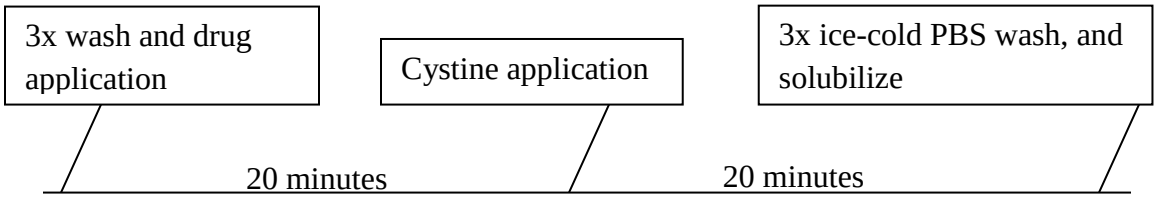
C6 glioma cell cultures

C6 glioma cells (ATCC, Manassas, VA) were grown and propagated in F-12K medium supplemented with 10% heat inactivated fetal bovine serum in 25cm^2 tissue flasks. For *in vitro* cystine uptake experiments, cells were lifted with trypsin (0.25% trypsin/0.53mM EDTA in HBSS), and plated in poly-lysine coated 24-well tissue culture

plates at a density of ~2 to $3x10^5$ cells/well. Cells were allowed to grow to confluence (~3 DIV) prior to testing for cystine uptake.

Acute regulation of cystine uptake in vitro

All experiments were performed in a 37ºC bath. As illustrated below, cells were washed 3x with 1mL warm HEPES buffered saline solution, vacuumed to 150 μL, and 250 μL of the drug was added to bring to the indicated concentration. Cells incubated for 20 minutes with the drug, then a small aliquot, 10 μL, of HEPES buffered saline solution was added that brought the concentration of [^{14}C]cystine (Perkin Elmer, Waltham, MA) and TBOA to 1 μM and 10 μM, respectively. Cells incubated for another 20 minutes. The uptake experiment was rapidly terminated by wash 3x with 1mL of ice-cold PBS, vacuumed dry, and 500 μL 0.1% SDS added to solubilize the cells. Resultant lysate was analyzed by scintillation counter and counts were normalized to the mean of the control



Time Dependent Glutamate Release in vitro

C6 glioma cells cultured as mentioned above were washed 3x with 1mL HEPES buffered saline solution. 10μL aliquots were collected at 0, 2.5, 5, 10, 20, 40, 80, and 160 minute increments. Each aliquot was measured for glutamate content by HPLC.

Time Dependent Cystine Uptake in vitro

C6 glioma cells cultured as mentioned above were wash 3x with 1mL HEPES buffered saline solution and [^{14}C]cystine (1μM) was added to the media. Cells were

washed 3x with 1mL ice-cold PBS, solubilized with 500μL 0.1% SDS, and measured for cystine by scintillation counter at 0, 2.5, 5, 10, 20, 40, 80, or 160 minutes.

Quantification of Glutamate

The concentration of glutamate in culture was quantified by comparing peak heights from samples and external standards using HPLC coupled to fluorescence detection. Precolumn derivatization of glutamate with opthaldehyde was performed using a Shimadzu LC10AD VP autosampler. The mobile phase consisted of 13% acetonitrile, 100 mM Na_2HPO_4, and 0.1 mM EDTA, pH 5.90. Glutamate was separated using a reversed-phase column (4 μm; 140 × 6.0 mm; Phenomenex, Torrance, CA), and detected using a Shimadzu 10RF-AXL fluorescence detector with excitation and emission wavelengths of 320 and 400 nm, respectively.

Quantification of Dopamine

Dopamine concentrations were quantified by comparing peak heights from samples and external standards using HPLC coupled to electrochemical detection. The mobile phase consisted of 15% acetonitrile, 10% methanol, 150 mM NaH2PO4, 4.76 mM citric acid, 3 mM SDS, and 50 μM EDTA, pH 5.6. Dopamine was separated using a reversed-phase column (3 μm; 80 × 3.2 mm; ESA Inc., Bedford, MA) and detected using an ESA Coulochem II detector coupled to two electrodes set at −0.075 V and +0.25 V, respectively.

Prefrontal cortical surgery

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 300–350 g were individually housed in a temperature-controlled colony room with a 12-h light/dark cycle with food and water available *ad libitum*. The housing conditions and care of the rats was

in accordance with the Animal Welfare Act, and all procedures were approved by the Marquette University IACU Committee. Rats included in the microdialysis studies were anesthetized using pentobarbital (50 mg/kg, IP) with atropine sulfate (1 mg/kg, IP) pretreatment to limit tracheobronchial secretions. Bilateral guide cannula (20 gauge, 14 mm; Plastics One, Roanoke, VA) were implanted using coordinates (+3.1 mm anterior and ±1.0 mm mediolateral to Bregma, and −0.75 mm ventral from the surface of the skull at a 6° angle from vertical) derived from (Paxinos and Watson, 1986). Rats were then given at least 5 days to recover from surgery prior to testing.

In Vivo Microdialysis

Microdialysis experiments were conducted as described previously (Baker et al., 2008). Briefly, removable probes were inserted through the guide cannulae into the prefrontal cortex. The next day, dialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.15% phosphate-buffered saline, pH 7.4) was pumped through the probes at 1 μl/min for at least 3 h; afterwards 20 min samples were collected before and after treatment with N-acetylcysteine (90mg/kg, IP). Dopamine concentrations were quantified by comparing peak heights from samples and external standards using HPLC coupled to electrochemical detection. The mobile phase consisted of 15% acetonitrile, 10% methanol, 150 mM NaH_2PO_4, 4.76 mM citric acid, 3 mM SDS, and 50 μM EDTA, pH 5.6. Dopamine was separated using a reversed-phase column (3 μm; 80 × 3.2 mm; ESA) and detected using an ESA Coulochem II detector coupled to two electrodes set at −0.075 V and +0.25 V, respectively.

Histology

Rats included in the microdialysis studies were given an overdose of pentobarbital (60 mg/kg, IP), and the brains were fixed by intracardiac infusion of 0.9% saline followed by 2.5% formalin solution. Brains were removed and stored in 2.5 % formalin for at least seven days prior to sectioning. The tissue was then blocked and coronal sections (100 μm) were cut and stained with cresyl violet to verify probe placements.

Statistical analyses

SPSS software (version 16) was used to perform the statistical analyses. When comparing between two treatments, a Student's *t* test was performed. Two way analysis of variance (ANOVA) was used with more than one between subject factors. Data collected over time were analyzed using analysis of variance (ANOVA) with sulfasalazine as a between subjects measure and time (20-min interval) as a repeated factor. *Post hoc* comparisons were conducted using t-tests when only two data points were compared, or with Tukey HSD with multiple data points.

Results

Differential cystine uptake in neurons and glia in culture

Figure 2.1 illustrates the differential uptake of cystine in primary mouse cortical culture. Pure neurons or pure glia were cultured and [^{14}C]cystine transport was measured. A two-way ANOVA with cell type and treatment as between subjects factors revealed a significant main effect of cell type ($F_{1,63}$=139.045, p<0.001), treatment ($F_{3,63}$=79.043, p<0.001), and a significant interaction between the two ($F_{3,63}$=43.311, p<0.001). The cystine glutamate exchange inhibitor (S)-4-carboxyphenylglycine was used to determine the system x_c^- specific portion of uptake, and D-aspartate was used to determine the system X_{AG}-specific portion of uptake. *Post hoc* analyses with treatment as a variable in neuronal culture yielded significant contributions of CPG-dependent uptake (ANOVA: $F_{3,31}$=20.193, p<0.001; Tukey HSD p<0.001) and D-aspartate-dependent uptake (Tukey HSD, p<0.001). Interestingly, *post hoc* analyses with treatment as a variable in glial culture revealed significant contributions of CPG-dependent uptake (ANOVA: $F_{3,31}$=69.105, p<0.001; Tukey HSD, p<0.001), but not for D-aspartate-dependent uptake (Tukey HSD, p=0.120).

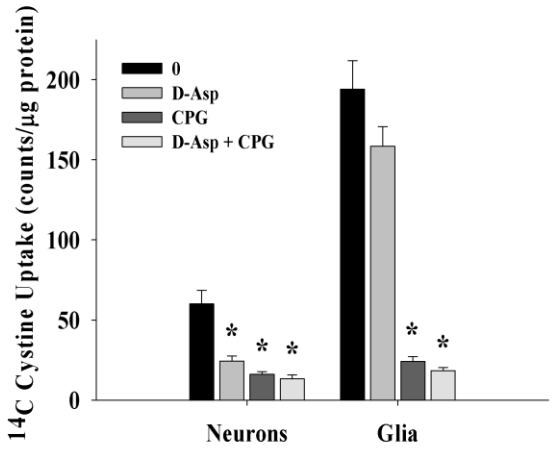


Figure 2.1. System x_c^- is expressed primarily on astrocytes. Primary mouse cortical cells were cultured for two weeks and measured for cystine uptake in the presence or absence of the cystine-glutamate exchange inhibitor (S)-4-carboxyphenylglycine (CPG) or the system X_{AG} inhibitor D-aspartate. A two-way ANOVA with cell and treatment as between subjects factors reveal a significant main effect of cell culture ($F_{1,63}$=139.045, p<0.001), treatment ($F_{3,63}$=79.043, p<0.001), and a significant interaction between the two ($F_{3,63}$=43.311, p<0.001). *Significant difference from control.

Cystine transport as a valid model for measuring cystine-glutamate exchange

The above experiments indicate that glia, likely astrocytes, is the primary cell type expressing cystine-glutamate exchange. Further, neurons express multiple sources of cystine transport in addition to system x_c-. C6 glioma cells were utilized in the following experiments because they express high levels of cystine-glutamate exchange with diminished system X_{AG} (Ye et al., 1999). These cells were used to determine if glutamate release or cystine transport is the optimal measure of system x_c- activity. Figure 2.2 illustrates that glutamate accumulates in the media over time even in the absence of exogenous cystine. A two-way ANOVA with time (0 – 160 min) as a repeated measure and SSZ as between subjects factors yielded significant main effects of time ($F_{7,47}$=144.808, p<0.001), SSZ ($F_{1,47}$=27.483, p<0.001), as well as a significant interaction ($F_{7,47}$=6.416, p<0.001). *Post hoc* analyses reveal that there was a significant increase in glutamate release in the absence of cystine at multiple time points (Tukey HSD, p<0.05). This was not significantly altered by the inhibition of system x_c- (i.e., SSZ) (Tukey HSD, p<0.001) indicating that most of the glutamate accumulating in the media is not due to cystine-glutamate exchange. These data demonstrate that one issue with measuring glutamate to reflect system xc- is that this approach is influenced by

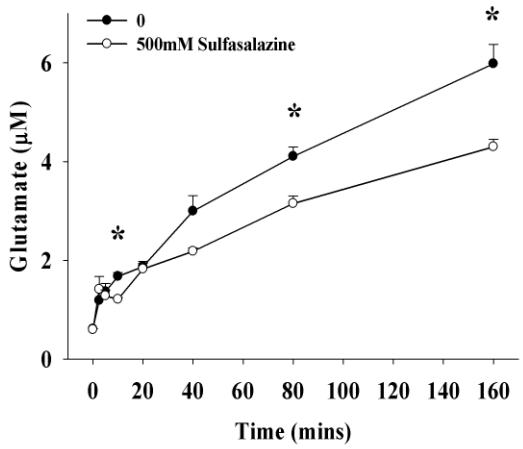


Figure 2.2. Cystine-glutamate exchange as assessed by the measurement of extracellular glutamate. C6 glioma cells were cultured, washed with HEPES buffered saline solution, and either measured for extracellular glutamate. In the presence of the cystine-glutamate exchange inhibitor sulfasalazine (0 or 500μM), glutamate release was significantly different for the 10, 80, and 160 minute time points. *Significant difference from SSZ treated (*t* test, p<0.05).

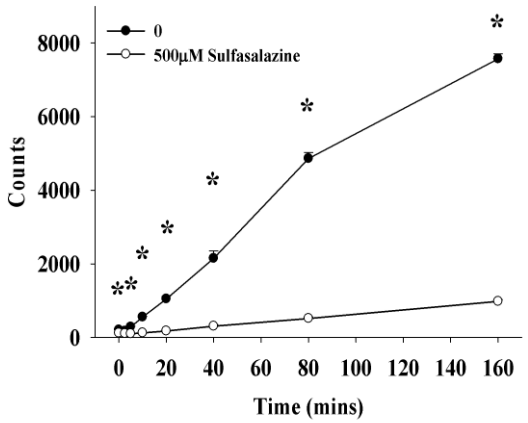


Figure 2.3. Cystine-glutamate exchange as assessed by the measurement of uptake of radiolabeled cystine. C6 glioma cells were cultured, washed with HEPES buffered saline solution, and measured for [^{14}C] cystine uptake. In the presence of the cystine-glutamate exchange inhibitor sulfasalazine (0 or 500μM), cystine transport was significantly different at most of the time points. *Significant difference from SSZ treated (*t* test, $p<0.05$).

other mechanisms contributing to glutamate homeostasis, and this may present signal-noise concerns.

Figure 2.3 illustrates that [^{14}C]cystine (1μM) transport significantly increased over time. A two way ANOVA with time and SSZ as between subject factors yielded significant main effects of time ($F_{7,44}=625.350$, $p<0.001$), SSZ ($F_{1,44}=1841.993$, $p<0.001$), as well as a significant interaction ($F_{7,44}=401.081$, $p<0.001$). Further, at 20 minutes, there was a significant difference, when compared to 0 minutes, in cystine uptake in the absence (Tukey HSD, $p<0.05$) but not the presence (Tukey HSD, $p=0.846$) of SSZ, as well as a significant difference between the two at 20 minutes ($t_4=11.145$, $p<0.001$). Given that cystine transport was significantly diminished in the absence of cystine-glutamate exchange (i.e. in the presence of SSZ), these findings suggest that cystine transport is a more reliable measure of cystine-glutamate exchange in culture.

Cystine-glutamate exchange in vitro is regulated by protein kinase A (PKA)

Previous studies have shown that cystine-glutamate exchange can be regulated by PKA in tissue punches (Baker et al., 2003) and *in vitro* (Tang and Kalivas, 2003). Our next goal was to determine whether a similar regulation occurs in C6 cells, in part to evaluate the suitability of using these cells to study the regulation of system xc- activity.

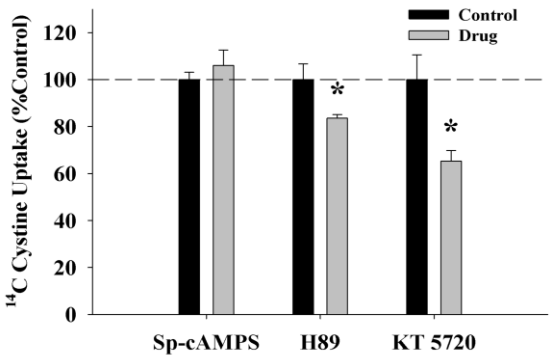


Figure 2.4. PKA regulates cystine-glutamate exchange in culture. C6 glioma cells were grown in culture and pretreated for 20 minutes with drugs that regulate PKA activity. Stimulating PKA with Sp-cAMPS had no significant effect on cystine transport (100μM; t_{29}=0.8100, p=0.425). Inhibiting PKA with H89 decreased cystine transport (10μM; t_6=0.2.4050, p=0.05), as well as with the more specific PKA inhibitor KT 5720 (100nM; t_6=3.0200, p<0.05). *Significant difference from control (*t* test, p<0.05).

Figure 2.4 illustrates regulation of cystine-glutamate exchange *in vitro* as measured by [^{14}C]cystine uptake in C6 glioma cells. Cystine transport was unaffected by stimulating protein kinase A (PKA) activity with the cAMP analog Sp-cAMPS (0 or 100 μM; t_{29}=0.8100, p=0.425). Interestingly, inhibiting PKA with H89 (0 or 10μM) showed a significant decrease in cystine uptake (10μM; t_6=0.2.4050, p=0.05). We also examined the effect of KT5720, a more potent and selective allosteric inhibitor of PKA. KT5720 significantly decreased cystine transport (0 or 100nM; t_6=3.0200, p<0.05). The drug concentrations used were based on previous publications for their use *in vitro*: SP-cAMPS (Song et al., 2006, Zhang et al., 2009, Zhang et al., 2010), H89 (Park et al., 2009), and KT572 (Russo et al., 2009). The lack of an effect of Sp-cAMPs creates concerns regarding the utility of using C6 cells to study the regulation of system xc-.

Cystine-glutamate exchange regulates dopamine signaling in the cortex

An alternative to using C6 cells is to use cortical cultures (Liu et al., 2009, Liu et al., 2011, Rush et al., 2012). Interestingly, similar to ex vivo punches (Baker et al., 2003), system xc- activity in these cells are also regulated by PKA (Tang and Kalivas, 2003), thereby supporting the use of these cells for the study of system x_c-. Because these cells are harvested from a region other than the nucleus accumbens, we sought to determine

whether system x_c- in the cortex regulates neurotransmission in this region in a manner that is similar to the nucleus accumbens. To do this, we examined the impact of the cysteine prodrug N-acetylcysteine on dopamine signaling. The reason for sampling dopamine rather than glutamate is that extracellular dopamine levels are known to be entirely neuronal in nature, thereby providing a more clear assessment of the synaptic activity. Figure 2.5 illustrates that stimulating cystine-glutamate exchange with N-acetylcysteine (NAC; 90 mg/kg, IP) decreased extracellular dopamine levels in the prefrontal cortex *in vivo*; a finding that is similar to what we have observed in the nucleus accumbens. A repeated measures ANOVA yielded a significant effect of time ($F_{8,24}$=6.609, p<0.001), suggesting cystine-glutamate exchange regulates neurotransmission in the cortex.

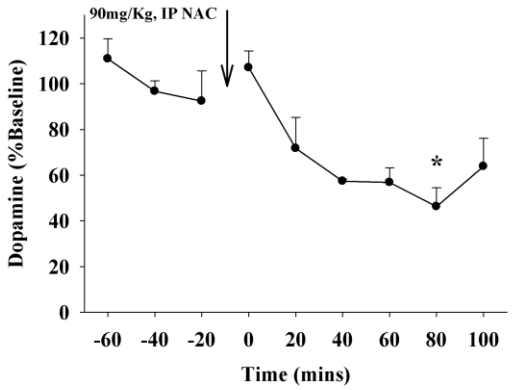


Figure 2.5. Stimulating cystine-glutamate exchange decreases dopamine in the prefrontal cortex. Acute treatment with N-acetylcysteine (90mg/kg, IP) decreases extracellular dopamine in the prefrontal cortex. A repeated measures ANOVA yielded a significant effect of time ($F_{8,24}$=6.609, p<0.001). *Significant difference from last baseline sample (Tukey HSD, p<0.05).

Discussion

The aim of this chapter was to determine the cell type and substrate for measuring cystine-glutamate exchange *in vitro*. Primary mouse cortical cultures, both pure neuronal and pure glial, were measured for [^{14}C]cystine uptake. The majority of cystine transport occurred in astrocytes as opposed to neurons. In addition, neurons expressed transport by system X_{AG} and system x_c-, whereas astrocytes only expressed transport by system x_c-.

These findings suggest that cystine-glutamate exchange is expressed primarily on astrocytes, and that these cells maintain a cleaner measure of system x_c^- activity per measure of protein in culture.

The C6 glioma cell line was utilized due to the fact that these cells express cystine-glutamate exchange but have decreased glutamate transporter function (Ye et al., 1999). We chose to measure glutamate release in the absence of exogenous cystine and found that extracellular glutamate increased both in the presence and absence of cystine-glutamate exchange despite application of exogenous cystine, suggesting that an alternate mechanism contributes to the accumulation of glutamate. One possible contribution to extracellular glutamate is glutathione cycling. When glutathione is released, it is metabolized into its amino acid components, among them glutamate and cysteine, which can be auto-oxidized into cystine. However, the primary source of intracellular cystine, the rate limiting step to glutathione synthesis, is cystine-glutamate exchange (Kranich et al., 1998). Therefore, blockade of cystine-glutamate exchange by sulfasalazine should inhibit increases in glutamate by glutathione cycling. Future experiments should investigate the source of extracellular glutamate.

We found that [^{14}C]cystine uptake significantly increase over time in a cystine-glutamate dependent manner. Important to note is that glutathione cycling may also contribute to confounds in cystine transport experiments. For example, as mentioned previously, cystine that is transported into the cell is utilized to form glutathione. This glutathione is then released via MRP-1, which could result in an underestimate of the total amount of cystine-glutamate exchange Therefore, it is important to utilize the shortest time possible to isolate the functional assay to cystine-glutamate exchange.

An important question regarding the utility of using C6 cells to learn about the regulation of system x_c- in vivo is the degree to which we can replicate in C6 cells what is known about in vivo system xc- regulation. To do this, we examined the impact of increasing PKA activity since this has been shown to regulate cystine-glutamate exchange in ex vivo punches as well as mixed cortical cultures (Baker et al., 2003, Tang and Kalivas, 2003). Stimulating protein kinase A (PKA) activity was unable to alter cystine transport. There are at least two interpretations of this data set. The first is that these cells exhibit tonic PKA activity at a level that is not observed in vivo or in primary cultured cells. Alternatively, this could indicate that PKA does not modulate system xc- activity in C6 cells. Interestingly, our results indicate that system xc- activity was increased in C6 cells following the activation of PKA, which supports the former interpretation. Regardless, either interpretation would indicate differential regulation of system xc- in C6 cells relative to either primary cultures or ex vivo tissue punches. This greatly detracts from the utility of these cells for the experiments in this thesis. Therefore, for future studies we utilized primary cultures.

Mixed cortical cultures are a common model for measuring regulation of cystine-glutamate exchange (Jackman et al., 2010, Liu et al., 2011, Rush et al., 2012). However, we are particularly interested in system x_c- in the nucleus accumbens core, given deficits in cystine-glutamate exchange in the nucleus accumbens core contribute to cocaine-seeking (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Knackstedt et al., 2010, Moussawi et al., 2011b). Therefore, we wanted to determine if cystine-glutamate exchange regulates neurotransmission in the cortex as it does in the nucleus accumbens. Figure 2.4 illustrates that stimulating cystine-glutamate exchange decreases extracellular

dopamine in the prefrontal cortex. This finding, coupled with previous demonstration that cystine-glutamate exchange by system x_c^- is maintained by PKA and PKC in mixed cortical culture (Tang and Kalivas, 2003), suggests that we should be able to measure regulation by neurotransmitter signaling in this culture. Cystine-glutamate exchange by system x_c^- has previously been shown tonically regulated by a number of growth factors including interleukin-1β (Jackman et al., 2010) and fibroblast growth factor-2 (Liu et al., 2011) in addition to acute regulation by protein kinase A (Baker et al., 2003) and protein kinase C (Tang and Kalivas, 2003). This reveals potential regulation by neurotransmitters that are linked to PKA or PKC, such as dopamine. Because we are interested specifically in changes that may account for reduced activity following repeated cocaine, in the following chapter we investigate regulation of cystine-glutamate by dopamine signaling.

CHAPTER 3

Regulation of system x_c^- by dopamine

Introduction

Extrasynaptic glutamate is a pool of extracellular glutamate contributed to by astrocytic mechanisms such as cystine-glutamate exchange by system x_c^- that regulates neurotransmission (Baker et al., 2002). Cystine-glutamate exchange is altered in multiple disorders including schizophrenia (Baker et al., 2008, Lavoie et al., 2008, Dean et al., 2009, Dean et al., 2010, Dean et al., 2011) and addiction (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Moussawi et al., 2011b). Interestingly, both disorders are considered hyperdopaminergic disorders based on their association with dopamine neurotransmission and altered behavior. This raises the possibility that system x_c^- is regulated by dopamine.

Previous findings suggest that cystine-glutamate exchange is regulated by PKA and PKC (Baker et al., 2003, Tang and Kalivas, 2003). Interestingly, dopamine receptors are G-protein coupled (Beaulieu and Gainetdinov, 2011). Particularly, D2-like dopamine receptors are G_i- and D1-like dopamine receptors are G_s-coupled, which means stimulating leads to respective inactivation and activation of PKA. The aim of this chapter is to determine if dopamine receptor signaling regulates cystine-glutamate exchange. We examine the regulation of system x_c^- by dopamine, nonspecific dopamine agonists, D1-like, and D2-like dopamine receptor agonists. We hypothesize that D1-like dopamine receptors increase, and D2-like dopamine receptors decrease cystine-glutamate exchange activity based on their respective activation and inactivation of PKA activity.

Materials and Methods

Animals

These experiments utilized male and female Sprague Dawley rats (Harlan, Indianapolis, IN). Female rats arrived at 14 days gestation. Rats were individually housed in a temperature-controlled colony room with a 12-h reversed light/dark cycle. Housing conditions and experimental protocols were approved by the Marquette University Institutional Animal Care and Use Committee and carried out according to the NIH Guide for the Care and Use of Laboratory Animals (revised 1996).

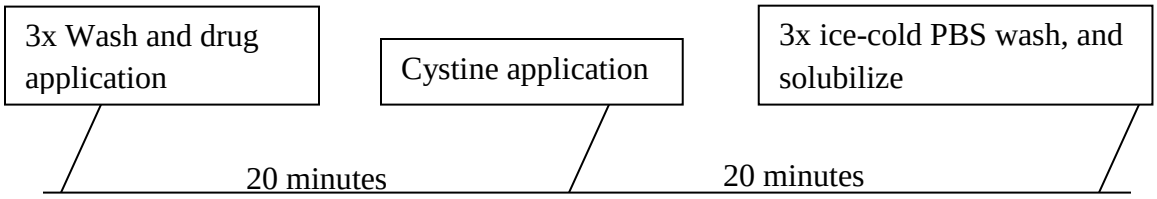
Rat mixed cortical cultures

Mixed cortical cell cultures containing glial and neuronal cells from rats were prepared from fetal (15–16 day gestation) rats as previously described (Lobner, 2000), with some exceptions. Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin at a density of ~$6x10^5$ cells/well in Neurobasal medium supplemented with 10% fetal bovine serum and 1% Glutamax-I (Life Technologies). Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. All efforts were made to minimize animal suffering and reduce the number of animals used.

Acute regulation of cystine-glutamate exchange in vitro

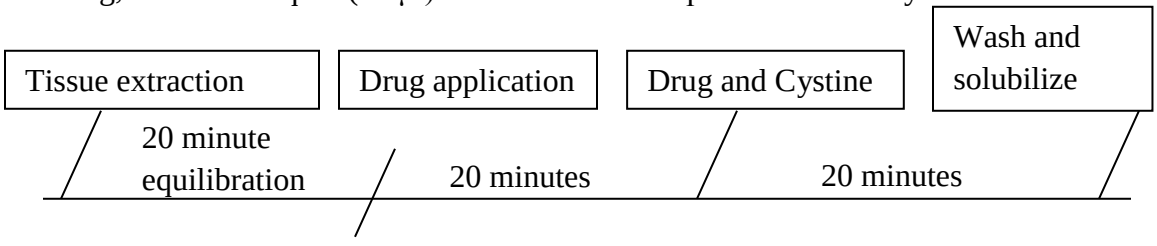
All experiments were performed in a 37ºC bath. As illustrated below, cells were washed 3x with 1mL warm HEPES buffered saline solution, vacuumed to 150 μL, and 250 μL of the drug was added to bring to the indicated drug concentration. Cells incubated for 20 minutes with the drug, then a small aliquot, 10 μL, of HEPES buffered saline solution was added that brought the concentration of [^{14}C]cystine (Perkin Elmer, Waltham, MA) and TBOA to 1 μM and 10 μM, respectively. Cells incubated for another

20 minutes. The uptake experiment was rapidly terminated by wash 3x with 1mL of ice-cold PBS, vacuumed dry, and 500 μL 0.1% SDS added to solubilize the cells. Resultant lysate was analyzed by scintillation counter and counts were normalized to the mean of the control group.



Regulation of cystine-glutamate exchange ex vivo

As illustrated below, tissue punches (1.25 mm diameter; Stoelting, Wood Dale, IL) were collected from the nucleus accumbens core (figure 1.4) and incubated at 37°C for ~30 min on a nylon mesh platform, submerged beneath 2 mm of standard buffer (Lobner and Lipton, 1993); the standard buffer contained 124 mM NaCl, 3.0 mM KCl, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 4 mM glucose, equilibrated with 95% O_2, 5% CO_2, pH 7.4. After a 20 min wash, the tissue was incubated for 20 min in standard buffer containing dopamine, SKF38393, or apomorphine and the cystine-glutamate exchange blocker sulfasalazine (0 or 500 μM). The tissue was transferred to standard buffer containing the drug treatment, [^{14}C]cystine (1 μM), and DL-TBOA (10 μM) to prevent uptake via X_{AG}. After 20 min incubation, the tissue punches were washed four times in ice-cold PBS and dissolved in 250 μl of 1% SDS. One aliquot (175 μl) was used to measure [^{14}C]cystine uptake by scintillation counting; a second aliquot (50 μl) was used to assess protein content by the BCA method.



Statistical analyses

SPSS software (version 16) was used to perform the statistical analyses. When comparing between two treatments, a Student's *t* test was performed. Two way analysis of variance (ANOVA) was used with more than one between subject factors (such as SCH23390 and intake condition). Experiments with multiple concentrations of one treatment utilized ANOVA. *Post hoc* comparisons were conducted using t-tests when only two data points were compared, or with Tukey HSD with multiple data points.

Results

D2-like dopamine receptors do not regulate cystine-glutamate exchange in vitro

PKA regulates cystine-glutamate exchange *ex vivo* (Baker et al., 2003) and *in vitro* (Tang and Kalivas, 2003). In chapter 2, we showed that inhibiting PKA activity *in vitro* decreased system x_c- activity. D2-like dopamine receptors are G_i-coupled and therefore decrease adenylyl cyclase and PKA activity (Beaulieu and Gainetdinov, 2011). We examined the ability of D2-like receptors to decrease cystine-glutamate exchange. Figure 3.1 illustrates that the selective D2 receptor agonist quinpirole (0 – 100μM) was unable to alter [^{14}C]cystine transport in rat mixed cortical cultures (ANOVA: $F_{5,47}$=0.500, p=0.774). Drug concentrations were selected based on previous findings (Li et al., 2006, Guo et al., 2010). Quinpirole was selected due to its affinity for D2-like versus D1-like dopamine receptors (K_m = 4.8nM for D2 and 1.9μM for D1) over other available agonists such as bromocriptine (K_m = 8nM for D2 and 440nM for D1).

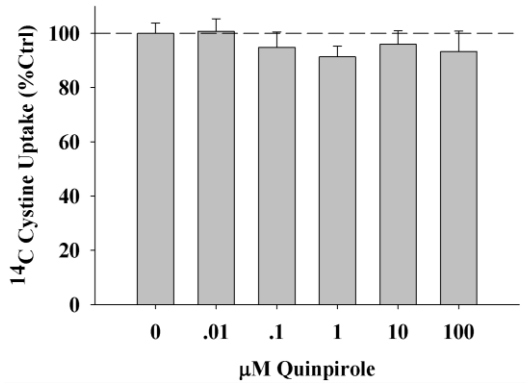


Figure 3.1. D2 receptor stimulation did not regulate cystine-glutamate exchange *in vitro*. Primary rat mixed cortical cultures were made from embryonic dissection (E15-E17) and cultured for 14 days. Pretreatment with the selective D2 agonist quinpirole (0 – 100μM) yielded no significant change in [^{14}C]cystine uptake ($F_{5,47}$=0.500, p=0.774).

Regulation of cystine-glutamate exchange by D1-like dopamine receptors

We also examined the ability of G_s-coupled D1-like dopamine receptors to regulate cystine-glutamate exchange. Figure 3.2 illustrates regulation of cystine-glutamate exchange by D1-dopamine receptors *in vitro*. Acute application of SKF38393 (0 – 1μM) increased [^{14}C]cystine transport in mixed cortical cultures from rat (ANOVA: $F_{3,39}$=3.587, p<0.05). *Post hoc* analyses show a significant effect of SKF38393 at the 0.1 μM concentration (Tukey HSD, p<0.05). The concentration of SKF38393 was selected based on previous findings (Dumont et al., 2004, Gomes et al., 2004).

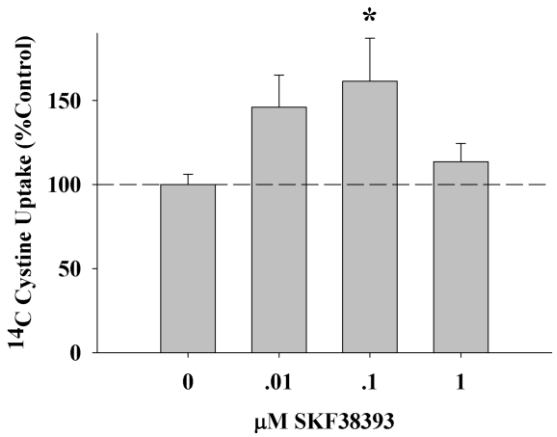


Figure 3.2. D1 dopamine receptor stimulation increased cystine-glutamate exchange *in vitro*. Primary rat mixed cortical cultures were made from embryonic dissection (E15-E17) and cultured for 14 days. Pretreatment with the D1 agonist SKF38393 (0 – 1μM) significantly increased [^{14}C]cystine uptake ($F_{3,39}$=3.587, p<0.05). *Significant difference from control (Tukey HSD, p<0.05).

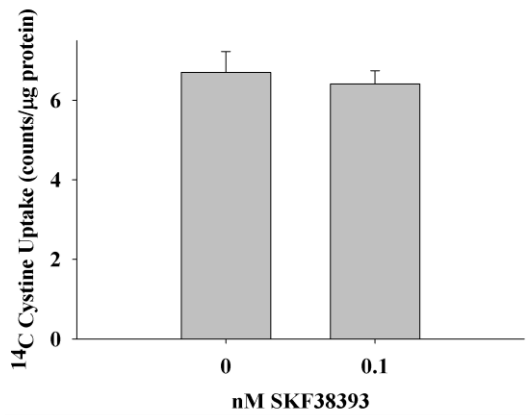


Figure 3.3. The D1 agonist SKF38393 does not increase cystine transport in the nucleus accumbens core *ex vivo*. 1mm x 1mm tissue punches from the nucleus accumbens core were extracted from drug naïve animals and incubated in slice buffer, pretreated with SKF38393 (0 or 100nM), then treated with SKF38393 and [^{14}C]cystine (1μM). A *t*-test yielded no significant effect of drug (t_9=0.450, p=0.664).

Cystine-glutamate exchange by system x_c- is diminished in the nucleus accumbens core of animals withdrawn from chronic cocaine. Therefore, we wanted to determine of D1-like dopamine receptors regulate cystine-glutamate exchange in *ex vivo* tissue punches from the nucleus accumbens core. Figure 3.3

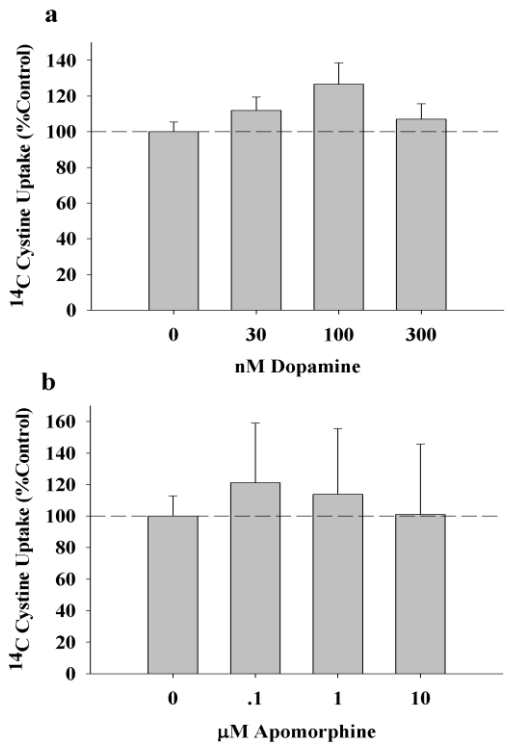


Figure 3.4. Nonspecific dopamine receptor stimulation did not regulate cystine-glutamate exchange in the nucleus accumbens core *ex vivo*. Tissue punches from the rat nucleus accumbens core were pretreated with (**a**) dopamine (0 – 300nM), (**b**) apomorphine (0 – 10µM), None of which had a significant effect on [^{14}C]cystine transport.

illustrates lack of regulation by SKF3839 (0 or 100nM) *ex vivo* (*t* test, t_9=0.450, p=0.664). One explanation for this finding is that there may be differences in dopamine receptor-dependent regulation of system xc- between primary cultures and ex vivo punches. The nature of the difference may be in terms of drug potency indicating that either higher or lower concentrations of SKF38393are needed to fully evaluate the effect of D1-like receptors on system xc- activity in ex vivo punches. Alternatively, the differences may be more substantive indicating differential regulation at the level of the receptor, which could complicate the interpretation of even the D2 data. Given that the rational for these experiments stems from the hypothesis that dopamine may regulate system xc-, additional characterization involved ex vivo punches and dopamine.Figure 3.4a illustrates that dopamine was not capable of altering cystine-glutamate exchange in *ex vivo* tissue punches from the nucleus accumbens core. We did observe a non-significant trend, suggesting that we were not able to fully stimulate dopamine receptors. There are two interpretations of this data set. First, dopamine may exert mild, insignificant effects on system xc- activity. Alternatively, the modest effect may be due to the elimination of dopamine since dopamine can be cleared by organic

cation transporters (OCT-3) and dopamine transporters (DAT) or inactivated by oxidation and metabolism by monoamine oxidase. Therefore, we looked at nonspecific stimulation of dopamine receptors by apomorphine, which would not be cleared or eliminated in a manner that may complicate the interpretation of the dopamine experiment. Figure 3.4b illustrates that apomorphine was not capable of altering cystine-glutamate exchange. The concentration of apomorphine used was based on previous findings eliciting effects in astrocytes (Li et al., 2006). ANOVAs run for [^{14}C]cystine transport yielded no significant effect of dopamine (3.4a; 0 – 300nM; $F_{3,33}$=2.014, p=0.131) or apomorphine (3.4b; 0 – 10μM; $F_{3,28}$=0.734, p=0.542).

Discussion

The aim of this chapter was to determine if diminished cystine-glutamate exchange during withdrawal from cocaine self-administration can be attributed to altered dopamine signaling (Bradberry and Roth, 1989, Xia et al., 1992, Cerruti et al., 1994, De Vries et al., 1999, Alleweireldt et al., 2002, Bachtell et al., 2005, Ben-Shahar et al., 2007). Cystine-glutamate exchange has previously been shown regulated by protein kinase A (Baker et al., 2003, Tang and Kalivas, 2003). D2-like dopamine receptors are linked to G_i signaling, decreased adenylyl cyclase activity, and cAMP production [for review see (Missale et al., 1998)]. Therefore, we hypothesized that D2 dopamine receptor stimulation would decrease cystine-glutamate exchange activity. Contrary to our expectations, the selective D2-like dopamine receptor agonist quinpirole was unsuccessful at altering cystine transport *in vitro*. This finding was surprising giving that others have shown regulation of cystine-glutamate exchange by receptors also linked to G_i, such as Group II metabotropic glutamate receptors (Xi et al., 2002a). In the

mentioned publication, stimulating and inhibiting Group II receptors decreased and increased, respectively, extracellular glutamate in the nucleus accumbens in a cystine-glutamate exchange dependent manner. Aside from targeting D2 receptors, our experiment differs in that it was performed in mixed cortical culture. A likely explanation for the lack of regulation of cystine-glutamate exchange by D2-like receptors is the level of expression of these receptors in the developing central nervous system. In particular, previous studies have suggested that in the developing rat cerebral cortex, D2 receptor expression does not reach maximal levels until postnatal day 35 (Tarazi and Baldessarini, 2000). In this study, they showed that postnatal day 14, similar to the age of the mixed cortical culture, rat showed no significant increase in D2 receptor expression than day 7.

We examined regulation of cystine-glutamate exchange by D1-dopamine receptors. D1-like dopamine receptors are coupled to G_s, and therefore activate PKA,leading us to hypothesize that targeting D1-like dopamine receptors would regulate system x_c- activity. Stimulating D1-like dopamine receptors *in vitro* significantly increased cystine transport, suggesting that D1-like dopamine receptors regulate cystine-glutamate exchange. Cystine-glutamate exchange by system x_c- is diminished in the nucleus accumbens core of animals withdrawn from chronic cocaine. Therefore, we wanted to determine of D1-like dopamine receptors regulate cystine-glutamate exchange in *ex vivo* tissue punches from the nucleus accumbens core. The D1 dopamine receptor agonist, SKF38393 did not alter cystine transport in the nucleus accumbens core *ex vivo*.The fact that D1-like receptor stimulation regulated cystine-glutamate exchange *in vitro* but not in tissue punches from the nucleus accumbens core is likely attributed to a number of differences between the systems. *In vitro* cultures were acquired from the

cortex, whereas the tissue punches are from the nucleus accumbens. Further, *in vivo* tissue punches were acquired from adult males, whereas the culture system originally derives from embryonic day 16, and are utilized 14 days later. Receptor expression and signaling develops over time in the rat. For example, GABA receptors act in a more stimulatory manner in early development due to elevated internal chloride concentrations in immature neurons (Pfeffer et al., 2009) and NMDA receptor subunit expression changes through early development (Wenzel et al., 1997). As with D2 receptors in the developing rat cortex, D1 receptor expression does not reach its maximum until at least postnatal day 60, which postnatal day 14 at significantly lower levels (Tarazi and Baldessarini, 2000). However, though there was no direct comparison between D1 and D2 receptors, the data presented in this publication suggest a higher expression of D1 receptors over D2 receptors; a potential reason for regulation of cystine-glutamate exchange by D1, but not D2 receptors in mixed cortical culture.

In tissue punches, afferent fibers are dissected out, leaving only cell bodies from the region from which the tissue was taken and removing synaptic signaling. Conversely, in mixed cortical culture neuronal signaling remains intact, revealing the possibility that D1-like receptor activation occurred on the cortical neurons, leading to release of neuronal signaling that may act on astrocytes. Cortical neurons, such as glutamatergic, project to the nucleus accumbens. Therefore it is possible that cortical activation, perhaps by D1-like dopamine receptor stimulation, may lead to downstream regulation of cystine-glutamate exchange in the nucleus accumbens. In support of this, previous investigators have found that a single activation of the prefrontal cortex can restore basal glutamate by cystine-glutamate exchange in the nucleus accumbens of cocaine-withdrawn animals

(Berglind et al., 2009). While we can report that stimulation of D1-like receptors in the nucleus accumbens core was unable to regulate cystine-glutamate exchange, it is possible that dopamine in the prefrontal cortex regulates cystine-glutamate exchange in the nucleus accumbens core. To address this, similar to previous experiments (Berglind et al., 2009), future studies should attempt to stimulate D1-like receptors in the prefrontal cortex and measure cystine-glutamate exchange *in vivo*. Predicted findings are likely that stimulation would lead to an increase in basal glutamate. Alternatively, it is possible that basal cystine-glutamate exchange is difficult to elevate *in vivo*. In that instance, one may consider attempting to restore cocaine-induced diminished cystine-glutamate exchange by stimulation of D1-like receptors in the prefrontal cortex.

Our assumption is that regulation by dopamine is dependent on dopamine receptor activation, yet in this system, it may result from non-receptor mediated actions. For example, cystine-glutamate exchange can be upregulated in the presence of oxidative stress (Allen et al., 2002, Dun et al., 2006, Lewerenz et al., 2006). Further, dopamine can induce oxidative stress either by auto-oxidation into dopamine quinones (LaVoie and Hastings, 1999) or by eventual formation of cysteinyl-dopamine (Rabinovic and Hastings, 1998). Thus, there may be alternate possibilities for dopamine to regulate cystine-glutamate exchange that are not specific to receptor stimulation. However, dopamine alone lacked an effect on cystine transport *ex vivo*. There are multiple sources of inactivation of dopamine signaling. Dopamine can be transported by organic cation transporter (OCT-3) (Wu et al., 1998) and dopamine transporters (DAT), and is metabolized by monoamine oxidase. Therefore, we tested a nonspecific dopamine receptor agonist that is not susceptible to normal metabolism. However, we were also

unable to alter cystine-glutamate exchange with the nonspecific dopamine agonist apomorphine. To this end, it may prove valuable to investigate a potential interaction of long-term increases in dopamine and altered cystine-glutamate exchange. Shih et al. have previously shown that excessive dopamine signaling *in vitro* can lead to activation of the Nrf2 transcription factor and eventual increase in expression of cystine glutamate exchange (Shih et al., 2007). Assuming this is the case, then one would assume that animals exhibiting elevated dopamine levels due to cocaine-withdrawal would also exhibit elevated levels of cystine-glutamate exchange by system x_c-. Therefore, future experiments may investigate this proposed pathway, DA > Nrf2 > system x_c-, in cocaine-withdrawn animals.

Our inability to regulate cystine-glutamate exchange by dopamine receptors in the nucleus accumbens core suggests that dopamine does not contribute to diminished cystine-glutamate exchange during withdrawal from chronic cocaine. In the following chapter we investigate the ability of a novel neuropeptide, pituitary adenylyl cyclase activating polypeptide (PACAP) to regulate cystine-glutamate exchange because it has previously been shown to regulate glutamate homeostasis in the striatum (Figiel and Engele, 2000).

CHAPTER 4

Regulation of system x_c^- by pituitary adenylyl cyclase activating polypeptide

Introduction

Glutamate homeostasis is altered following withdrawal from cocaine self-administration due to reduced cystine-glutamate exchange by system x_c^- (Pierce et al., 1996, Baker et al., 2003); which can be attributed in part to reduced expression of xCT, the functional subunit of system x_c^- (Knackstedt et al., 2010). We are interested in determining if neurotransmitters and peptides play a role in regulating cystine-glutamate exchange. Interestingly, a novel neuropeptide, pituitary adenylyl cyclase activating polypeptide (PACAP) is likely co-released with synaptic glutamate based on findings that it is produced in the cortex (Mikkelsen et al., 1994, Figiel and Engele, 2000) and is colocalized with vesicular glutamate transporter 2 (Engelund et al., 2010). PACAP has also been shown to regulate glutamate homeostasis through glutamate transporters such as GLT-1 (Figiel and Engele, 2000). Others have found that some treatments leading to an increase in GLT-1 expression have also increased expression of cystine-glutamate exchange (Knackstedt et al., 2010)

The aim of this chapter is to determine if PACAP signaling regulates cystine-glutamate exchange. We examine PACAP regulation of cystine transport *in vitro* and *ex vivo*. In chapter 3, we were unable to alter cystine-glutamate exchange with receptors known to couple to G_i and G_s. However, PAC1 receptors are coupled to G_s or G_q. G_q signaling stimulates Ca^{2+}-dependent protein kinase (PKC), also previously shown to regulate cystine-glutamate exchange (Tang and Kalivas, 2003). Three G-protein coupled receptors have relevant affinity for PACAP: PAC1, VPAC1, and VPAC2; with PAC1 having the highest affinity and selectivity for PACAP (Lam et al., 1990). PAC1 receptor signaling is linked to both G_s or G_q mediated signaling (Holighaus et al., 2011). Further,

xCT has consensus phosphorylation sites and is regulated by PKA and PKC as shown in chapter 2 and previously (Baker et al., 2003, Tang and Kalivas, 2003). Therefore, we hypothesize that PAC1R activation increases cystine-glutamate exchange based on its ability to stimulate both PKA and PKC activity (Holighaus et al., 2011).

Materials and Methods

Animals

All experimental protocols were approved by the Marquette University Institutional Animal Care and Use Committee and carried out according to the NIH Guide for the Care and Use of Laboratory Animals (revised 1996).

Rat mixed cortical cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15–16 day gestation) rat as previously described (Lobner, 2000), with some exceptions. Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin at a density of ~$6x10^5$ cells/well in Neurobasal medium supplemented with 10% fetal bovine serum and 1% Glutamax-I (Life Technologies). Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. Rats were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

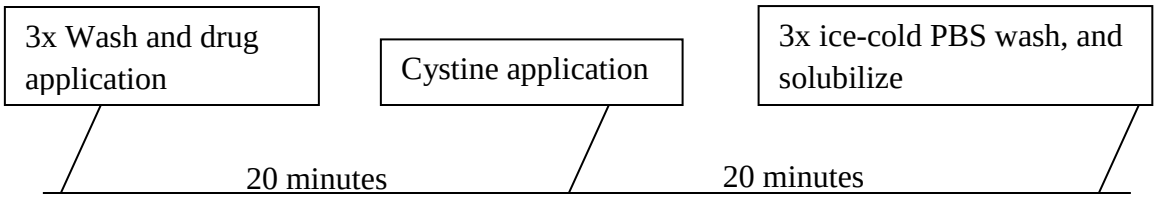
Mouse cortical cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15–16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Astrocyte enriched glial cultures were prepared as described for mixed cultures except they are from cortical tissue taken from post-natal day 1–3 mice (Choi et al., 1987, Schwartz and Wilson, 1992). Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Acute regulation of cystine uptake in vitro

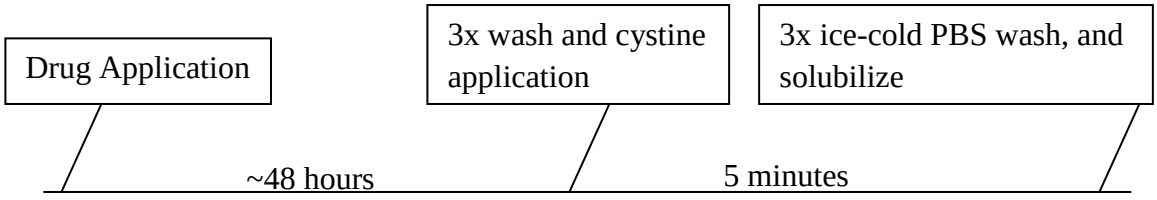
All experiments were performed in a 37ºC bath. As illustrated below, rat mixed cortical or differentiated striatal astrocyte cultures were washed 3x with 1mL warm HEPES buffered saline solution, vacuumed to 150 μL, then 250 μL of the drug was added to bring to the indicated concentration. Cells incubated for 20 minutes with the drug, then a small aliquot, 10 μL, of HEPES buffered saline solution was added that brought the concentration of [^{14}C]cystine (Perkin Elmer, Waltham, MA) and TBOA to 1 μM and 10 μM, respectively. Cells incubated for another 20 minutes. The uptake experiment was rapidly terminated by wash 3x with 1mL of ice-cold PBS, vacuumed dry,

and 500 μL 0.1% SDS added to solubilize the cells. Resultant lysate was analyzed by scintillation counter and counts were normalized to the mean of the control group.



Long-term regulation of cystine uptake in vitro

As illustrated below, mouse cortical cultures cells were treated with indicated drugs (; or 0 or 100 nM PACAP) for ~48 hours in growth culture media. For the PACAP antagonist (0 or 10 μM PACAP6-38) experiment, mixed cortical culture was used. For the PACAP38 experiment (0 or 100nM), pure glia were used. On test days, cells were washed 3x with 1mL HEPES buffered saline solution and vacuumed to 150 mL. Then, 250 μL of HEPES buffered saline solution containing cystine to bring the culture media to one of the following concentrations (CC denotes "cold" cystine, CC* denotes [^{14}C]cystine): 0.1 μM (0.1 μM CC*), 0.3 μM (0.3 μM CC*), 1 μM (1 μM CC*), 3 μM (1 μM CC + 2 μM CC*), 10 μM (1 μM CC + 9 μM CC*), or 30 μM (3 μM CC + 27 μM CC*) as previously described (Fogal et al., 2007). Five minutes later, uptake was rapidly terminated by washing 3x with 1mL of ice-cold PBS, 250 μL of 0.1% SDS was added and allowed to solubilize the cells. 175 μL was used for scintillation counting and 50 μL was used for measuring protein by the BCA method.



Aliquots of radiolabeled cystine at 0, 1, 3, and 10 pmoles were measured by scintillation counter, divided by the cystine content and averaged to determine the specific activity. Amount of cystine transport was then determined using the following equation:

$$\frac{\text{cellular cpm}}{\left[\frac{\text{spec. activity}}{([S]+[S^*])/[S^*])}\right]}$$

([S] = concentration of cold cystine, [S*] = concentration of hot cystine)

Cystine transport was normalized to protein to yield transport/μg protein.

In order to measure changes in Km or Vmax, uptake data was converted to a Hanes-Woolf plot by plotting velocity/[CC] against [CC]. For Hanes-Woolf plot, V_{max}=1/slope, and K_m= - x-intercept.

Surgeries

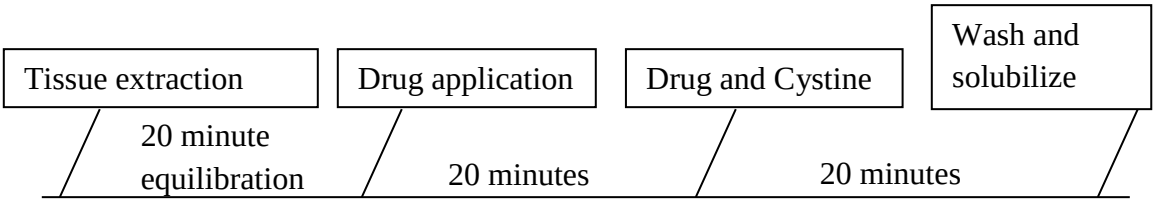
Rats were implanted with indwelling catheters under ketamine HCl (100 mg/kg, IP, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (2 mg/kg, IP, Lloyd Laboratories, Shenandoah, IA, USA) anesthesia. A silicon tubing catheter (Dow Corning Co., Midland, MI; 0.64 mm ID; 1.19 mm OD) was implanted such that it entered the jugular vein through the right posterior facial vein and terminated at the right atrium. The catheter was sutured to the vein at the entry point. The distal aspect of the catheter, which consisted of a 22-gauge guide cannula (Plastics One Inc., Roanoke, VA) attached with dental acrylic to a piece of polypropylene monofilament surgical mesh (Atrium Medical, Co., Hudson, NH), exited 2 cm posterior to the scapulae. Throughout the experiment, catheters were filled daily with a heparin solution (83 i.u./ml; Elkins-Sinn, Inc., Cherry Hill, NJ) and capped when disconnected from the leash/delivery line assembly.

Cocaine Self-Administration Training

Self-administration occurred in operant conditioning chambers (ENV-008CT, MED-Associates Inc., St Albans, VT, USA) housed in sound attenuating cubicles (ENV-016M, MED-Associates Inc.) and equipped with two retractable levers, two stimulus lights, and a water bottle. At least five days after surgery, rats were food restricted with water available ad libitum; food restriction continued for the duration of the experiment (e.g., through reinstatement testing) and involved daily administration of 15 grams of rat chow in the late afternoon or immediately following an operant session. Rats were then placed into the operant conditioning chambers overnight and responses on the lever designated as active resulted in the delivery of food pellets under a fixed ratio 1 schedule of reinforcement. Daily food training continued until subjects received at least 150 food rewards in a session, which typically occurred following the first session. *During the acquisition phase of the experiment,* all rats underwent drug self-administration training during daily 2-hr sessions in which operant responses on the active lever were reinforced with an infusion of cocaine (0.5 mg/kg/200 μl IV, National Institute on Drug Abuse, Bethesda, MD, USA) under a fixed ratio 1 schedule of reinforcement. Each reinforced lever response resulted in the illumination of the stimulus light located above the active lever and was followed by a 25-s time-out period. Responding on a second, inactive lever located on the back wall was recorded but had no programmed consequences. Acquisition of cocaine self-administration was operationally defined as < 10% variation in daily responding over at least three consecutive sessions. *During the maintenance phase of the experiment,* rats were assigned to self-administer cocaine under high-intake conditions (1.0 mg/kg/200 μl IV; 6-hr/day for 11 days).

Regulation of cystine-glutamate exchange ex vivo

Rats withdrawn from saline or high-intake (1.0 mg/kg/200 mL infusion, i.v., 6 h/d) cocaine self-administration were decapitated and the brains were rapidly extracted and cut into 1 mm coronal slices using a brain matrix. As illustrated below, tissue punches (1.25 mm diameter; Stoelting, Wood Dale, IL) were collected from the nucleus accumbens core (figure 1-4) and incubated at 37°C for ~30 min on a nylon bolting cloth platform, submerged beneath 2 mm of standard buffer (Lobner and Lipton, 1993); the standard buffer contained 124 mM NaCl, 3.0 mM KCl, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 4 mM glucose, equilibrated with 95% O_2, 5% CO_2, pH 7.4. After a 20 min wash, the tissue was incubated for 20 min in standard buffer containing PACAP (0 – 1 nM), or PACAP (0 or 1 nM) and the cystine-glutamate exchange blocker sulfasalazine (0 or 500 μM). The tissue was transferred to standard buffer containing [^{14}C]cystine (1 μM). DL-TBOA (10 μM) was also added to the incubation buffer to prevent uptake via X_{AG}. After 20 min incubation, the tissue punches were washed four times in ice-cold PBS and dissolved in 250 μl of 1% SDS. One aliquot (175 μl) was used to measure [^{14}C]cystine uptake by scintillation counting; a second aliquot (50 μl) was used to assess protein content by the BCA method.



Statistical analyses

SPSS software (version 16) was used to perform the statistical analyses. When comparing between two treatments, a Student's *t* test was performed. Two way analysis

of variance (ANOVA) was used with more than one between subject factors (such as PACAP6-38 and sulfasalazine). Experiments with multiple concentrations of one treatment utilized ANOVA. *Post hoc* comparisons were conducted using t-tests when only two data points were compared, or with Tukey HSD with multiple data points.

Results

Regulation of xc- by pituitary adenylyl cyclase activating polypeptide

We examined the regulation of cystine-glutamate exchange by PACAP. Figure 4.1 illustrates that exogenous PACAP was not capable of altering [^{14}C]cystine uptake in mixed cortical cultures from rat (ANOVA: $F_{4,39}$=0.830, p=0.515). Because cortical cultures produce PACAP endogenously (Figiel and Engele, 2000), we looked to determine if blocking PACAP signaling *in vitro* would alter [^{14}C]cystine uptake.

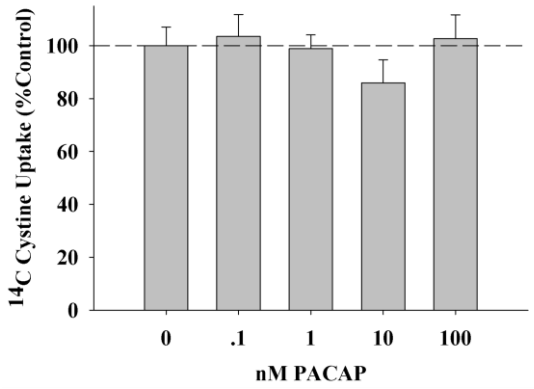


Figure 4.1. Acute treatment of PACAP in rat mixed cortical cultures does not affect cystine-glutamate exchange. Primary rat mixed cortical cultures were pretreated with PACAP (0 – 100nM) and tested for cystine uptake. An ANOVA revealed no significant change from control (ANOVA: $F_{4,39}$=0.830, p=0.515).

Figure 4.2a illustrates that acute blockade of PAC1Rs with the specific antagonist PACAP6-38 reduced cystine-glutamate exchange (ANOVA: $F_{3,55}$=14.551, p<0.001). *Post hoc* analyses reveal a significant effect of 10μM PACAP6-38 (Tukey HSD, p<0.001). We then looked to determine if this decrease in [^{14}C]cystine uptake was specific to cystine-glutamate exchange. Figure 4.2b illustrates that PACAP signaling regulates [^{14}C]cystine uptake by system x_c-. A two-way ANOVA with PACAP6-38 (0 or 10μM) and sulfasalazine (0 or 500μM) as between subjects factors yielded significant main effects of PACAP6-38 ($F_{1,12}$=8.190, p<0.05) and SSZ ($F_{1,12}$=233.707, p<0.001), as well as an interaction between the two ($F_{1,12}$=8.190, p<0.05). *Post hoc* analyses confirmed a significant effect of PACAP6-38 in the absence (t_6=2.898, p<0.05), but not the presence of SSZ (t_6<0.001,

p=1.000). The concentration of PACAP6-38 that induced the significant effect on cystine-glutamate exchange is the same as used in a previous publication (Figiel and Engele, 2000).

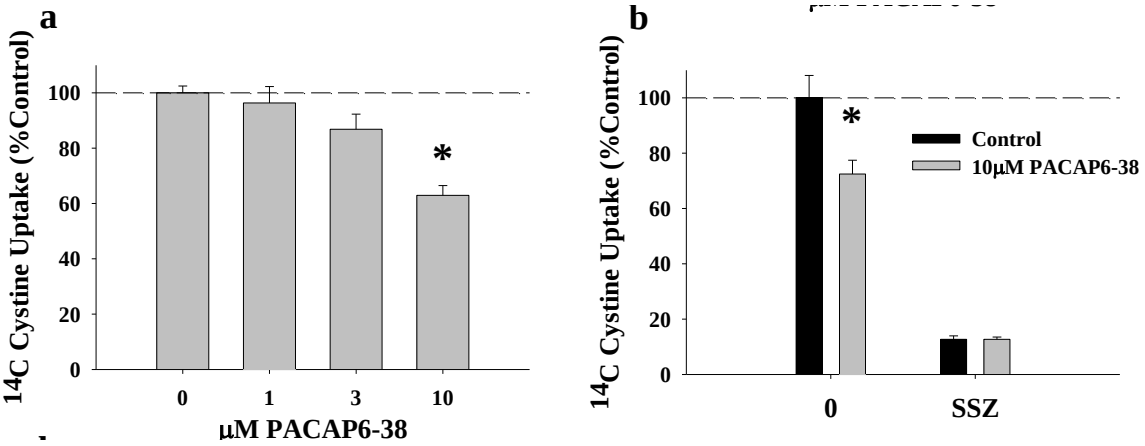


Figure 4.2. PACAP Regulates cystine-glutamate exchange. (**a**) Acute treatment with the PAC1R antagonist, PACAP6-38, showed a significant regulation of cystine uptake in rat primary mixed cortical cultures (ANOVA: $F_{3,55}$=14.551, p<0.001; 10μM, Tukey HSD, p<0.001). (**b**) Addition of sulfasalazine (0 or 500 μM) shows PACAP6-38 regulation of cystine-glutamate exchange in *ex vivo* tissue punches (t_6=2.898, p<0.05).

Protein Kinase C signaling is necessary for PACAP regulation of cystine-glutamate exchange

Because PAC1 receptors can stimulate PKA and PKC (Holighaus et al., 2011), we examined the role of PKA and PKC signaling in PACAP regulation of cystine-glutamate exchange. Figure 4.3a illustrates that PACAP regulation of cystine-glutamate exchange is not dependent on PKA activity. We treated primary culture with PACAP6-38 (0 or 10μM) and the PKA activator dBcAMP (0 or 100μM). An ANOVA yielded a significant effect of treatment ($F_{3,41}$=9.749, p<0.001). *Post hoc* analyses revealed a significant effect of PACAP6-38 (Tukey HSD, p<0.001). However, there was no significant difference of PACAP6-38 in the presence and absence of dBcAMP (Tukey HSD, p=0.084).

We also examined the role of PKC on PACAP regulation of cystine-glutamate exchange. Figure 4.3b illustrates that PACAP regulation of system x_c- activity is dependent on protein kinase C (PKC) signaling. We treated primary culture with PACAP6-38 (0 or 10μM) and the PKC activator phorbol 12-myristate 13-acetate (PMA; 0 or 1μM). An ANOVA yielded a significant effect of treatment ($F_{3,41}$=11.573, p<0.001). *Post hoc* analyses revealed a significant decrease with PACAP6-38 (Tukey HSD, p<0.05). In addition, we measured a significant difference in cells treated with PACAP6-38 in the presence or absence of PMA (Tukey HSD, p<0.001). These findings suggest that PKC activity is necessary for PACAP regulation of system x_c-.

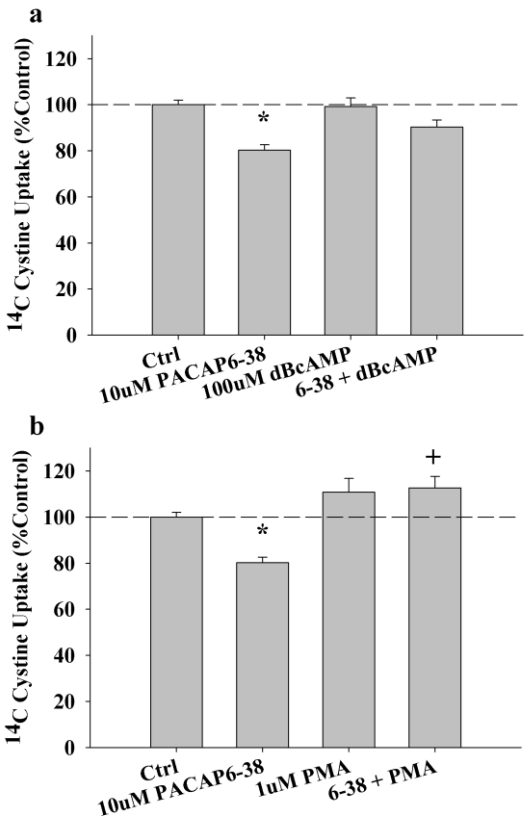


Figure 4.3. PACAP regulates cystine-glutamate exchange in a PKC-dependent manner. Primary rat mixed cortical cultures were pretreated with the noted compounds. Pretreatment with the PACAP receptor antagonist PACAP6-38 (10μM) significantly decreased cystine transport. This decrease was reversed with the PKC activator phorbol 12-myristate 13-acetate (PMA, 100nM), but not with the PKA activator dibutyryl cAMP (dBcAMP, 100μM). *Significant effect from control. +Significant effect from PACAP6-38 alone.

PACAP regulation of system x_c- activity is altered in cocaine-withdrawn animals

Acute treatment with PACAP was unable to regulate cystine-glutamate exchange in culture because of endogenous PACAP release. If this is the case, then we should be able to appropriately stimulate PAC1R in *ex vivo* tissue punches given that afferent corticostriatal pathways are denervated. Therefore, we looked to determine if PACAP signaling regulates system x_c- activity in the nucleus accumbens core as it does *in vitro* by acute application of PACAP. Figure 4.4 illustrates that PACAP regulates cystine-glutamate exchange in nucleus accumbens core tissue punches. A two way ANOVA with PACAP (0 or 1nM) and the cystine-glutamate exchange inhibitor sulfasalazine (SSZ, 0 or 500μM) as between subjects factors yielded a significant main effect of SSZ ($F_{1,19}$=69.431, $p<0.001$) as well as an interaction between PACAP and SSZ ($F_{1,19}$=6.874, $p<0.05$), but no main effect of PACAP ($F_{1,19}$=1.032, $p=0.325$). When splitting the groups based on SSZ, a t test comparing the PACAP groups (0 or 1nM) revealed a significant effect of PACAP in the absence of SSZ (t_{10}=2.563, $p<0.05$), but not in the presence of SSZ (t_6=1.374, $p=0.218$).

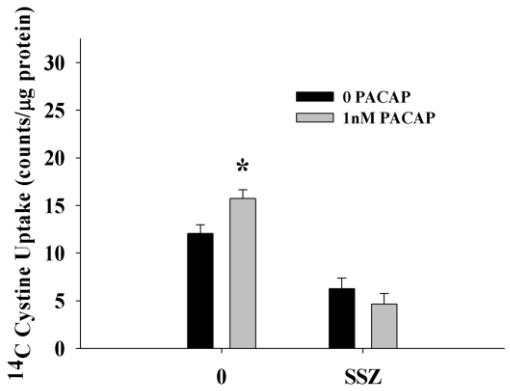


Figure 4.4. PACAP regulates cystine-glutamate exchange in the nucleus accumbens core. Pretreatment with PACAP (0 or 1nM) and SSZ (0 or 500μM) showed a significant effect of PACAP on [^{14}C]cystine transport by system x_c- in *ex vivo* tissue punches. *Significant difference from control.

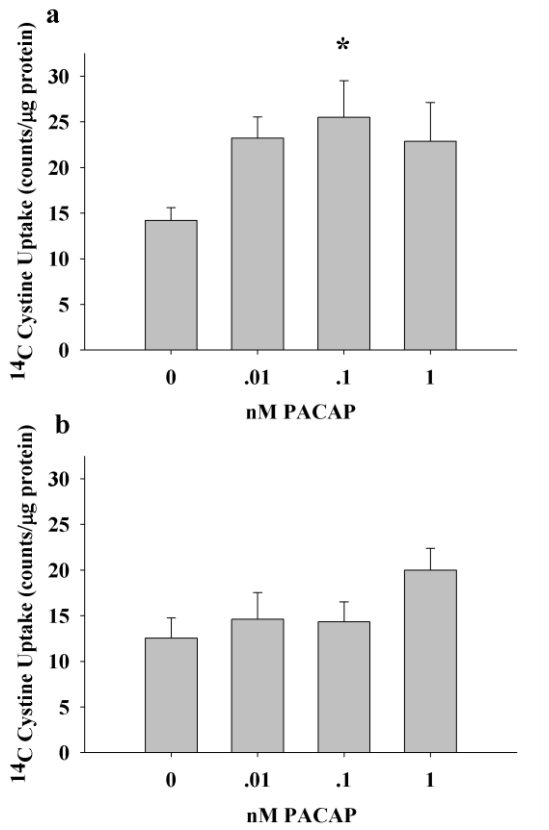


Figure 4.5. PACAP regulation of cystine-glutamate exchange in the nucleus accumbens core is diminished in cocaine-withdrawn animals. Rats withdrawn from saline (**a**) or cocaine (**b**) self-administration are represented. Drug naïve animals showed increases in cystine transport with acute treatment of PACAP ($F_{3,42}$=2.876, p<0.05). Animals withdrawn from cocaine self-administration did not respond to PACAP ($F_{3,18}$=1.670, p=0.216). *Significant difference from control (Tukey HSD, p<0.05).

We then examined the ability of PACAP to regulate cystine-glutamate exchange in animals withdrawn from cocaine self-administration. Pretreatment with PACAP resulted in a significant effect on [^{14}C]cystine transport for drug naïve animals (Figure 4.5a; ANOVA: $F_{3,42}$=2.876, p<0.05), but not for cocaine-withdrawn animals (Figure 4.5b; ANOVA: $F_{3,18}$=1.670, p=0.216). *Post hoc* analyses reveal a significant effect at the 0.1nM concentration for drug naïve animals (Tukey HSD, p<0.05).

Tonic PACAP signaling regulates the capacity of cystine-glutamate exchange

The previous findings show that acute PACAP signaling regulates cystine-glutamate exchange. However, there is a tonic decrease in cystine-glutamate exchange during withdrawal from cocaine self-administration. We are interested in what leads to this long-term diminished system x_c- activity. In order to determine if a long-term

decrease in PACAP signaling leads to diminished cystine-glutamate exchange, mixed cortical cultures were treated with the PAC1R antagonist PACAP6-38 for 48 hours. Figure 4.6 illustrates that long-term blockade of PACAP signaling results in a decrease in cystine-glutamate exchange. A *t* test yielded a significant difference between controls and PACAP6-38 (t_{30}=3.287, p<0.001). These findings suggest that a decrease in PACAP signaling may contribute to diminished basal cystine-glutamate exchange in cocaine-withdrawn animals.

We wanted to determine if long-term regulation of x_c- by PACAP changes the K_m or V_{max} of cystine transport. In order to do this, we treated pure glial cultures with PACAP for 48 hours and measured the kinetic activity of [^{14}C]cystine transport. An ANOVA with concentration of cystine as a repeated measure and PACAP (0 or 100 nM) as a between subjects measure yielded a significant interaction (figure 4.7a;

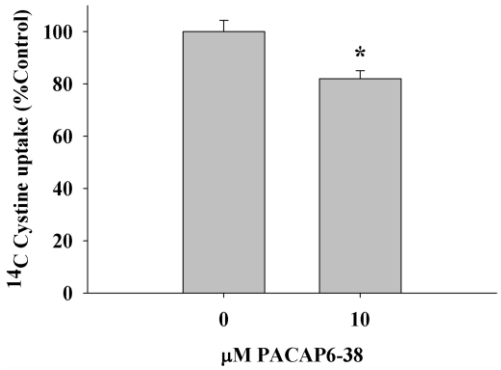


Figure 4.6. Tonic PACAP signaling regulates cystine-glutamate exchange. Blocking PAC1R receptors for 48 hours in mixed cortical culture decreases overall cystine uptake (t_{30}=3.287, p<0.001).

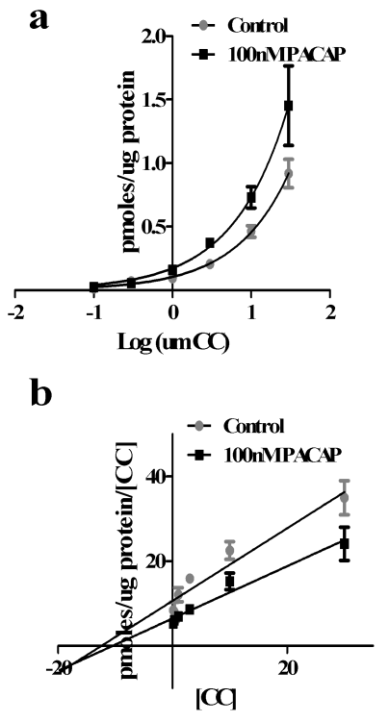


Figure 4.7. Tonic PACAP signaling regulates the capacity (V_{max}) of cystine-glutamate exchange on glia. (**a**) Plotting [CC] against the velocity portrays the raw transport data. (**b**) The Hanes-Woolf plot allows for interpretation of the data. 48 hour incubation of pure glia by PACAP increases the capacity, or V_{max}, of the transporter (ANOVA: $F_{1,44}$=4.259, p<0.05).

$F_{5,50}$=2.966, p<0.05). Converting to and analyzing as a Hanes-Woolf plot revealed a significant increase in the V_{max} (figure 4.7b; ANOVA: $F_{1,44}$=4.259, p<0.05) with PACAP.

Discussion

The aim of this chapter was to determine if PACAP signaling regulates cystine-glutamate exchange. PACAP has been previously shown to regulate glutamate homeostasis by stimulating receptors on astrocytes (Figiel and Engele, 2000). In this chapter, data is presented suggesting that PACAP also regulates glutamate homeostasis by stimulating cystine-glutamate exchange.

We first found that acute treatment with PACAP in mixed cortical culture was unable to alter cystine transport. Interestingly, cortical neurons produce and release PACAP *in vitro* (Figiel and Engele, 2000). Therefore, we hypothesized that blocking endogenous PACAP signaling would be able to decrease cystine-glutamate exchange. To test this, we treated mixed cortical culture with the selective PAC1R antagonist, PACAP6-38 and found this was capable of decreasing cystine transport in mixed cortical culture. There is concern that the concentration of PACAP6-38 required (10μM) to significantly decrease cystine-glutamate exchange is orders of magnitude higher than the effective concentrations of PACAP (Holighaus et al., 2011). However, the concentration of PACAP6-38 used was identical to that used by others (Figiel and Engele, 2000). In addition, our ability to regulate cystine-glutamate exchange in tissue punches from the nucleus accumbens core by PACAP further supports our findings.

Cystine-glutamate exchange is diminished in the nucleus accumbens core of cocaine-withdrawn animals (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008), contributing to cocaine-seeking. In order to determine if PACAP regulation of system x_c-

is altered in cocaine-withdrawn animals, we examined the capacity of PACAP to regulate cystine-glutamate exchange in tissue punches from the nucleus accumbens core of drug naïve and cocaine-withdrawn animals.

We found that cystine-glutamate exchange in animals withdrawn from high-intake cocaine self-administration is not regulated by PACAP, whereas it was regulated in drug naïve animals. Lack of phasic (acute) regulation does not fully explain the decrease in cystine-glutamate exchange we find in *ex vivo* tissue punches of cocaine-withdrawn animals, as seen in chapter 1. Therefore, it is likely that diminished tonic regulation by PACAP contributes to diminished cystine-glutamate exchange. This scenario is highly possible given that animals withdrawn from cocaine self-administration exhibit a form of hyprofrontality (Sun and Rebec, 2006), and PACAP is likely co-released with glutamate from the prefrontal cortex (Mikkelsen et al., 1994, Engelund et al., 2010) and possibly explains decreased GLT-1 expression in cocaine-withdrawn animals (Knackstedt et al., 2010) given that PACAP regulates GLT-1 expression (Figiel and Engele, 2000). Future studies should attempt to restore PACAP signaling in the nucleus accumbens core of cocaine-withdrawn animals in an effort to reverse the cocaine-induced decrease in cystine-glutamate exchange. In addition, future studies will examine the capacity of exogenous stimulation of PAC1R to alter cocaine-primed reinstatement in animals that exhibit augmented reinstatement.

Previous findings suggest that tonic PACAP signaling regulates glutamate homeostasis through glutamate transporters (Figiel and Engele, 2000). We found that long-term PACAP signaling regulates the maximal capacity of cystine transport *in vitro*, suggesting that basal PACAP contributes to the expression of system x_c-. What is

unknown is the mechanism by which long-term PACAP signaling regulates cystine-glutamate exchange. Previous studies have shown that PACAP can be considered a neuroprotectant (Onoue et al., 2002, Deguil et al., 2007, Deguil et al., 2010, Botia et al., 2011, Endo et al., 2011). The aforementioned studies fall short in determining the mechanism by which it acts as a neurprotectant. As mentioned previously, it is possible that neuronal signaling may contribute to cystine-glutamate exchange, a mechanism by which PACAP as a neuroprotectant can maintain system x_c-. PACAP regulates cystine-glutamate exchange, also a neuroprotectant through its eventual formation of the antioxidant glutathione (Kranich et al., 1998), therefore it is possible that PACAP-induced expression of cystine-glutamate is the mechanism by which the neuroprotectant is working, though future investigation is warranted.

Altogether, these findings suggest that PACAP regulates both short- and long-term activity of cystine-glutamate exchange, and reveals PACAP signaling as a novel target for cocaine addiction research.

PACAP regulation of cystine-glutamate exchange in vitro

PACAP has been shown to regulate glutamate homeostasis by increasing glial-glutamate transport (GLT-1) *in vitro* (Figiel and Engele, 2000). Interestingly, stimulation of PAC1R can lead to downstream effects including increases in cAMP and Ca^{2+} (Tanaka et al., 1996, Grimaldi and Cavallaro, 1999, Aoyagi and Takahashi, 2001, Mustafa et al., 2007), however the PACAP-mediated increases in GLT-1 were found to be protein kinase C (PKC) mediated (Figiel and Engele, 2000). PKC is activated by diacyl glycerol (DAG) and Ca^{2+} as a result of activation of G_q coupled receptors. This suggests that the PACAP-induced increases in GLT-1 expression were calcium mediated. Conversely,

others have found that PACAP only increases cAMP and not Ca^{2+} in astrocytes (Grimaldi and Cavallaro, 1999).

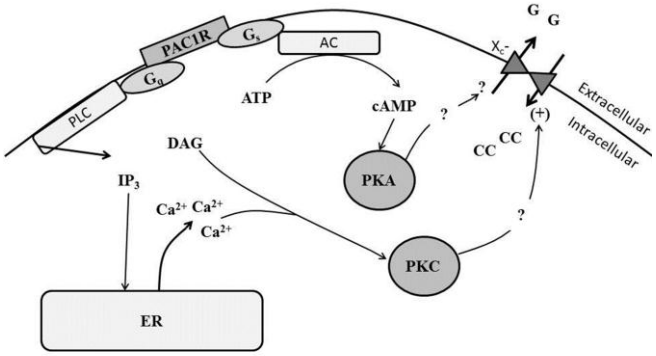


Figure 4.8. PACAP regulates system x_c^- through PKC. PAC1Rs activates G_q signaling, stimulating phospholipase C (PLC). PLC produces inositol trisphosphate (IP_3) and diacylglycerol (DAG) membrane lipids. IP_3 induces release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum (ER). Ca^{2+} and DAG activate protein kinase C (PKC). PKC increases activity of cystine-glutamate exchange. PLC, phospholipase C; IP_3, inositol trisphosphate; ER, endoplasmic reituculum; DAG, diacyl glycerol; PKC, protein kinase C; AC, adenylyl cyclase; PKA, protein kinase A.

In rat mixed cortical culture, we found that PACAP was unable to affect cystine transport. At first this was surprising giving the existing presence of PACAP signaling in basal mixed cortical cultures (Figiel and Engele, 2000). There could be two explanations for the inability of PACAP to stimulate cystine transport *in vitro*. First, there is enough basal signaling from neurons in these cultures that the concentration of PACAP added was not sufficient to overcome the endogenous signaling; though increasing the concentration of PACAP over that used in these experiments would lose specificity for the PAC1 receptor. Second, there may be intrinsic activity of the PAC1R that could not be overcome by adding more PACAP but could only be affected by the antagonist.

Pretreatment with the selective PAC1R antagonist, PACAP6-38 significantly decreased cystine transport in these cultures by affecting PKC signaling (figure 4.3b). At this point, we do not know if PACAP-induced activation of PKC acts directly on system x_c^- or through an intermediary. Future studies in our lab will look to determine if xCT,

the functional subunit of system x_c- is directly phosphorylated. To do this, point mutations at putative phosphorylation sites for xCT will be developed. The Mutated cDNA can then be transfected into a cell line that lacks significant levels of xCT such as neurons or HEK293 cells (Shih and Murphy, 2001). This method will allow the investigator to determine changes in both trafficking and kinetics, potentially revealing more novel and therapeutic targets for the treatment of cocaine addiction.

Altered Regulation of x_c- by PACAP in Cocaine-withdrawn Animals

Cystine-glutamate exchange is diminished in the nucleus accumbens core of animals withdrawn from cocaine self-administration (Baker et al., 2003, Moran et al., 2005, Moussawi et al., 2011b). We looked to determine if cystine-glutamate exchange is regulated by PACAP in drug naïve and cocaine-withdrawn animals. In *ex vivo* tissue punches, synaptic signaling is absent due to denervation of the efferent pathways; therefore, applying PACAP should have an effect on the PAC1R without the concern of overcoming endogenous PACAP signaling. We found that acute application of PACAP to *ex vivo* tissue punches from the nucleus accumbens core was able to significantly increase cystine-glutamate exchange in drug naïve but not cocaine-withdrawn animals.

It is possible that diminished PACAP regulation of cystine-glutamate exchange in cocaine-withdrawn animals may be due to diminished PACAP release. However, currently technology does not allow for accurate measurement of extracellular PACAP in the brain. In this experimental model, however, the neurons that project PACAP to the nucleus accumbens core are denervated, effectively eliminating endogenous PACAP signaling. Therefore, altered receptor signaling, either through receptor expression or signaling pathways, likely contributes to this effect. Further, PAC1 receptors are

expressed both on neurons and astrocytes (Grimaldi and Cavallaro, 1999), making it difficult to identify the source of PACAP regulation of cystine-glutamate exchange. As mentioned in chapter 3, it is possible that receptor activation on neurons could lead to regulation of cystine-glutamate exchange. Further studies may reveal the ability to differentially measure the expression of the receptor on neurons and astrocytes, which could allow an experimenter to further investigate the contribution of cocaine to altered PACAP signaling.

Pituitary adenylyl cyclase activating polypeptide (PACAP) regulates glutamate homeostasis by maintaining glial glutamate transport (Figiel and Engele, 2000) and cystine-glutamate exchange, as shown in this chapter. Animals withdrawn from cocaine self-administration show diminished cystine-glutamate exchange and basal extracellular glutamate in the nucleus accumbens (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Moussawi et al., 2011b). Though PACAP regulated cystine-glutamate exchange in the nucleus accumbens core of drug naïve animals, cocaine-withdrawn animals did not exhibit that effect. This suggests that basal extracellular glutamate is regulated in an acute manner by PACAP. Our findings are novel in that acute regulation of cystine-glutamate exchange is rarely investigated, PACAP regulation of cystine-glutamate exchange has never been reported, and certainly the deficit found in PACAP signaling in cocaine-withdrawn animals has never been discovered.

Figiel and Engele (2000) increased glial glutamate transport by long-term application of PACAP. We wanted to determine if system x_c^- responds in the same manner. Just as with glial glutamate transport, we found that 48 hour treatment of the PAC1R antagonist, PACAP6-38 led to a decrease in cystine transport in mixed culture.

To investigate further, we applied PACAP to pure glial cultures for 48 hours. Though there was no change in the binding affinity, K_m, we did find a significant increase in the full efficacy, or V_{max}. These findings suggest that PACAP signaling plays an extremely important role in glutamate homeostasis due to the fact that it regulates both the source of extrasynaptic, nonvesicular glutamate as well as the mechanism for clearance (Figiel and Engele, 2000). This likely effects the regulation of synaptic transmission as cystine-glutamate exchange regulates synaptic release (Baker et al., 2002), and that glutamate transport is necessary for synaptic clearance and maintaining the distinct pools of extracellular glutamate (Danbolt, 2001).

Chapter 1 showed that amount of cocaine-intake leads to differing levels of neuroplasticity. For example, animals withdrawn from high-intake cocaine self-administration exhibited augmented extracellular dopamine in the nucleus accumbens after a cocaine injection when compared to animals withdrawn from low-intake cocaine. It was mentioned earlier that these conditions reveal a more accurate assessment of neuroplasticity following chronic cocaine. The current studies compared drug naïve and animals withdrawn from high-intake cocaine self-administration, therefore it may prove valuable to repeat the current study to include animals withdrawn from low-intake cocaine self-administration conditions.

Historically, investigators have suggested that corticostriatal pathways regulate extracellular dopamine by targeting presynaptic terminals of midbrain dopaminergic neurons directly (Stewart, 2000, Schmidt et al., 2005, Thomas et al., 2008). Our findings suggest that a novel method of regulating dopamine release may converge at cystine-glutamate exchange. Not much is known as to the contribution of hypofrontality to the

neuropathology of addiction. We find that PACAP regulates cystine-glutamate exchange both tonic and acutely. As well, cystine-glutamate exchange regulates dopamine release, which contributes to elevated reinstatement. Therefore, diminished corticostriatal, and therefore PACAP signaling may contribute to augmented dopamine and relapse vulnerability.

CHAPTER 5

DISCUSSION

Compulsive cocaine seeking emerges after chronic use that is thought to be the direct result of drug-induced plasticity (Hyman and Malenka, 2001, Pierce and Vanderschuren, 2010). Cystine-glutamate exchange has been previously identified as a mechanism effected by chronic cocaine and contributes to multiple aspects of addiction (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Moussawi et al., 2011b). Interestingly, previous studies have found that targeting cystine-glutamate exchange an effective treatment for drug craving in humans (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Moussawi et al., 2011b). The purpose of the experiments in this dissertation was to better understand cystine-glutamate exchange by system x_c- in terms of the impact that diminished cystine-glutamate exchange has on behaviors used to model cocaine addiction, and its regulation at the cellular level to begin to understand how it may be reduced following repeated cocaine. Our primary findings were the following:

- Decreased basal extracellular glutamate due to diminished cystine-glutamate exchange contributes to augmented cocaine-evoked dopamine and heightened relapse vulnerability
- D1-like dopamine receptors regulate system x_c- activity in mixed cortical culture, but not tissue punches from the nucleus accumbens core
- PACAP acutely regulates cystine-glutamate exchange both in mixed cortical culture and in tissue punches from the nucleus accumbens core
- In animals withdrawn from cocaine self-administration, PACAP does not regulate system x_c- activity
- Long-term PACAP signaling regulates the maximal capacity of system x_c- suggesting a source of diminished cystine-glutamate exchange in cocaine withdrawn animals

Diminished system x_c- contributes to relapse vulnerability

We found that diminished cystine-glutamate exchange led to neuroplasticity and cocaine-seeking following withdrawal from cocaine self-administration. By manipulating conditions that restore basal glutamate and functional cystine-glutamate exchange, we were able to determine the contribution of altered extracellular glutamate in cocaine-primed reinstatement. Our findings show that diminished basal glutamate due to diminished cystine-glutamate exchange is essential to cocaine-primed reinstatement and cocaine-evoked extracellular glutamate.

Glutamate release into the nucleus accumbens core is necessary for cocaine-primed reinstatement (McFarland et al., 2003), therefore we hypothesized that animals exhibiting augmented cocaine-primed reinstatement would also show elevated cocaine-evoked extracellular glutamate. However, we did not detect a difference in cocaine-evoked glutamate despite the fact that animals receiving high-intake cocaine had diminished cystine-glutamate exchange. A possible explanation for this is findings by others that suggest uncoupling of presynaptic inhibitory Group II autorceptors in the nucleus accumbens core (Xi et al., 2002b). Conversely, we did find elevated cocaine-evoked dopamine in animals that consumed higher levels of cocaine. This was surprising as glutamate (McFarland et al., 2003) but not dopamine (McFarland and Kalivas, 2001, Anderson et al., 2003) is necessary for cocaine-primed reinstatement. Further, acute stimulation of cystine-glutamate exchange with N-acetylcysteine reversed the augmented cocaine-evoked dopamine response as well as decreased reinstatement. This suggests that diminished cystine-glutamate exchange contributes to augmented cocaine-evoked

dopamine. While glutamate signaling is necessary for cocaine-primed reinstatement, these findings reveal dopamine as a likely contributor to heightened relapse vulnerability.

Diminished cystine-glutamate exchange in cocaine-withdrawn animals: contribution of dysregulation by neurotransmission

In chapter 3, we examined the ability of dopamine to regulate cystine-glutamate exchange given that animals exhibiting augmented dopamine signaling also show heightened reinstatement. We did find a significant increase in [^{14}C]cystine transport in mixed cortical cultures treated with a D1-like receptor agonist. However, we found no regulation of cystine-glutamate exchange in nucleus accumbens core tissue punches by a D1-like dopamine receptor agonist. The differential regulation of system x_c- by D1 receptors was not expected. However, in tissue punches corticostriatal projections are eliminated, whereas in mixed cortical cultures cortical neuronal signaling remains intact. Therefore, it is possible that there is a contribution of dopamine regulation of corticostriatal signaling that regulates cystine-glutamate exchange. In support of this, findings by others suggest that acute stimulation of the prefrontal cortex by BDNF both restores basal glutamate in the nucleus accumbens and reduces cocaine seeking in cocaine-withdrawn animals (Berglind et al., 2007, Berglind et al., 2009). Our findings suggests the scenario that corticostriatal signaling regulates cystine-glutamate exchange, a phenomenon that could not be parsed out in a 1mm tissue punch from the nucleus accumbens core. Future experiments should investigate D1-like receptor stimulation in the prefrontal cortex and its contribution to cystine-glutamate exchange in the nucleus accumbens core. This idea is significant due to the fact that we showed regulation of

system x_c^- by the cortical derived neuropeptide pituitary adenylyl cyclase activating polypeptide (PACAP).

In addition to investigating regulation of x_c^- by D1-like dopamine receptors, we also looked into regulation by D2-like receptors. We were unable to alter system x_c^- activity by stimulating D2-like dopamine receptors *in vitro* even though these receptors are G_i-linked and we were able to decrease cystine-glutamate exchange by inhibiting PKA activity in glioma culture.

We also wanted to determine if nonspecific dopamine signaling regulates cystine-glutamate exchange. Oxidative stress is capable of increasing system x_c^- activity (Allen et al., 2002, Dun et al., 2006, Lewerenz et al., 2006). Therefore, we considered the possibility that dopamine may regulate cystine-glutamate exchange by non-receptor mediated mechanisms such as oxidative stress through metabolism or auto-oxidation (Rabinovic and Hastings, 1998, LaVoie and Hastings, 1999). However, dopamine alone lacked an effect on cystine transport *ex vivo*. Taking into account that dopamine can be inactivated through metabolism or uptake, we also tested apomorphine because it is a nonspecific dopamine receptor agonist that is not susceptible to normal metabolism. We did not find altered cystine-glutamate exchange in the presence of apomorphine. Further, there exists D1-D2 dopamine receptor heterodimers that are G_q-linked (Rashid et al., 2007), and binding of both the D1 and D2 receptor subunit is necessary for the downstream Ca^{2+} response. In support of our design, apomorphine is capable of binding to both receptor subtypes, suggesting that the heterodimer does not regulate cystine-glutamate exchange

We then decided to investigate a pituitary adenylyl cyclase activating polypeptide (PACAP) as a potential regulator of cystine-glutamate exchange. PACAP is likely co-released with glutamate based on the facts that it colocalizes with vesicular glutamate transporter 2 (VGLUT2) (Engelund et al., 2010) and that PACAP is produced in regions with glutamate projecting neurons such as the cortex (Mikkelsen et al., 1994, Figiel and Engele, 2000). As well, PACAP signaling regulates glutamate homeostasis by increasing glial glutamate transporter expression through a PKC-dependent mechanism (Figiel and Engele, 2000). We found that PACAP did indeed regulate system x_c- activity in mixed cortical cultures and tissue punches from the nucleus accumbens core, an effect dependent on PKC activity. More importantly, we found that cystine-glutamate exchange in the nucleus accumbens core of animals withdrawn from cocaine self-administration did not exhibit regulation of cystine-glutamate exchange by PACAP. These findings suggest that cystine-glutamate exchange is dysregulated by altered PACAP signaling in animals withdrawn from chronic cocaine.

As mentioned previously, PACAP mediates glutamate homeostasis by tonic regulation of GLT-1 function (Figiel and Engele, 2000). Interestingly, GLT-1 function has been previously shown diminished in the nucleus accumbens core of animals withdrawn from cocaine self-administration (Knackstedt et al., 2010). In the mentioned study, treatment with the β-lactam antibiotic, ceftriaxone restored GLT-1 and system x_c- in cocaine-withdrawn animals, suggesting that expression of GLT-1 and cystine-glutamate exchange are linked. Because PACAP tonically regulates GLT-1 function, we hypothesized that it also tonically regulates cystine-glutamate exchange. We found that long-term blockade of PACAP signaling in mixed cortical cultures that endogenously

produce PACAP resulted in a decreased in system x_c^- activity. We then looked to determine if long-term PACAP signaling regulates cystine-glutamate exchange particularly on astrocytes. When treating glial cultures with PACAP for 48 hours, we measured an increase in the maximal capacity of cystine-glutamate exchange.

Pituitary adenylyl cyclase activating polypeptide (PACAP) and its contribution to altered neurotransmission

PACAP is a novel neuropeptide likely co-released with synaptic glutamate in the nucleus accumbens core due to the fact that PACAP mRNA is found in regions that produce PACAP (Mikkelsen et al., 1994), is colabeled with vesicular glutamate transporter 2 (VGLUT2) (Engelund et al., 2010), and PAC1 receptors are found in the nucleus accumbens (Hannibal, 2002). This is significant because we found that PACAP regulates cystine-glutamate exchange in mixed cortical culture in a PKC-dependent manner. Given that cystine-glutamate exchange is diminished in the nucleus accumbens core in animals withdrawn from cocaine self-administration, we looked to determine if PACAP regulates system x_c^- activity in the nucleus accumbens core. We found that PACAP does regulate cystine-glutamate in the nucleus accumbens core. Interestingly, we found that PACAP regulation of cystine-glutamate exchange is diminished in animals withdrawn from cocaine self-administration.

Hypofrontality is a phenomenon described by decreased basal activity of the frontal cortical regions of the brain during a disorder such as cocaine addiction (Sun and Rebec, 2006) and schizophrenia (Jacquy et al., 1976, Buchsbaum and Wu, 1987). Interestingly, system x_c^- is diminished in both disorders (Baker et al., 2003, Baker et al., 2008). As mentioned previously, PACAP derives from cortical regions and is likely co-

released with synaptic glutamate. Therefore, if PACAP signaling is diminished due to hypofrontality, one may expect this is a major contributor to the decrease found in cystine-glutamate exchange (Baker et al., 2003, Moran et al., 2005, Kau et al., 2008, Knackstedt et al., 2010, Moussawi et al., 2011b) and GLT-1 (Knackstedt et al., 2010) in cocaine-withdrawn animals. Investigators may also benefit from investigation of PACAP signaling in the neuropathology of schizophrenia.

PAC1 receptors are both G_s- and G_q-coupled (Holighaus et al., 2011). However, we found that PACAP regulation of cystine-glutamate exchange is a PKC-dependent effect, implicating a G_q-coupled mechanism. In addition, we were unable to regulate system x_c- activity by a G_s-coupled receptor such as D1-like dopamine receptors. This is in line with previous findings that PACAP regulation of GLT-1 expression is dependent on PKC signaling. Another metabotropic receptor that is also coupled to G_q and is found on astrocytes is Group I metabotropic glutamate receptors, specifically mGluR5. If hypofrontality contributes to altered glutamate homeostasis due to diminished activation of PAC1 receptors, it is likely that diminished basal glutamate, both synaptic and through cystine-glutamate exchange, is insufficient to stimulate mGluR5. Therefore, future studies should investigate other G_q-coupled receptors, in particular mGluR5, and their role in altered glutamate homeostasis.

System x_c-: a novel convergence of corticostriatal and midbrain dopaminergic signaling

Historically, investigators have suggested that corticostriatal pathways regulate extracellular dopamine by targeting presynaptic terminals of midbrain dopaminergic neurons directly (Stewart, 2000, Schmidt et al., 2005, Thomas et al., 2008). Our findings suggest that a novel method of regulating dopamine release may converge at cystine-

glutamate exchange. Until now, not much is known as to the contribution of hypofrontality to the neuropathology of addiction. We find that the cortical-derived neuropeptide, PACAP regulates cystine-glutamate exchange both tonic and acutely. As well, cystine-glutamate exchange regulates dopamine release, which contributes to elevated reinstatement. Therefore, diminished corticostriatal signaling, in part through PACAP, may contribute to augmented dopamine release into the nucleus accumbens core and heightened relapse vulnerability.

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