Regulation of System Xc-by the Neuropeptide PACAP: Implications for Glutamate Transmission in Drug Addiction

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REGULATION OF SYSTEM X_C^- BY THE NEUROPEPTIDE PACAP: IMPLICATIONS FOR GLUTAMATE TRANSMISSION IN DRUG ADDICTION

by

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A Dissertation submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy

Milwaukee, Wisconsin

May 2017

ABSTRACT
REGULATION OF SYSTEM X_C^- BY THE NEUROPEPTIDE PACAP:
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IN DRUG ADDICTION

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Drug addiction is a chronic brain disorder characterized by heightened relapse susceptibility. Drug-induced aberrant glutamate signaling in corticostriatal circuitry contributes to behaviors in virtually every preclinical model of drug seeking and correlates with drug craving in human. Here, we propose that glutamate signaling is a product of integrated activity between neurons and astrocytes, such that disruptions within astrocytes can stem from abnormal neuronal signaling (e.g., altered corticostriatal firing) and be the source of additional disruptions in other neuronal circuits. The astrocytic mechanism studied in these experiments is system x_c^- (Sxc) since drug-induced changes to this non-vesicular glutamate release mechanism contribute to heightened relapse vulnerability in preclinical models of addiction. My first objective was to determine whether neurons or neuronal factors regulate Sxc activity in astrocytes (Chapter II and III) since this would illustrate the degree to which glutamate signaling involves integrated activity of multiple cell types. We found that neurons release a soluble factor that potently increases Sxc activity. Moreover, we discovered that the neuropeptide PACAP (pituitary adenylate cyclase-activating peptide) likely contributes to this effect since it upregulates Sxc activity in astrocytes (Chapters II and III). Next, we focused on the importance of PACAP regulation of Sxc to synaptic transmission in the nucleus accumbens (NAc), since this structure is highly implicated in drug addiction (Chapter IV). PACAP depressed synaptic transmission in NAc neurons projecting to the substantia nigra, an important efferent that encodes motivated behaviors. Interestingly, PACAP-induced control over synaptic transmission required enhanced Sxc activity. Given this, we then determined the behavioral impact of increasing PACAP signaling in the NAc on drug-seeking behavior (Chapter IV). Specifically, we found that microinfusion of PACAP into the NAc attenuated cocaine-primed reinstatement of drug seeking. Lastly, we determined that PACAP is expressed in corticostriatal projections to the NAc, and that endogenous PACAP is an unrecognized factor influencing relapse vulnerability (Chapter IV). Collectively, this dissertation reveals that a novel form of neuron-astrocyte communication, namely PACAP regulation of Sxc, is a critical link integrating the glutamate network that mediates motivated behavior.

ACKNOWLEDGEMENTS

Linghai Kong, B.S.

First, I thank my committee, Dr. David Baker, Dr. James Buchanan, Dr. Sujean Choi, Dr. Doug Lobner, and Dr. John Mantsch for their guidance and support. Particularly, I thank Dr. David Baker for accepting me into his lab, instilling in me knowledge and skills, and being a great mentor these years.

Many thanks to the Baker lab crew: to Nicholas Raddatz and Christopher Mueller for keeping the lab running, for constantly improving my English, for your technical support and help with virtually everything else; to former graduate students Dr. Aric Madayag and Dr. Victoria Lutgen, for introducing everything about the new lab to me and teaching me techniques; to current graduate student, Evan Hess, for your contribution to this work; to former undergraduate assistants, Tristan Brown, Patrick Budny, Wiley Yao, Lucy Christensen, Katie Grunewald and Derek Nicolas, Erik Van Newenhizen, much of this work would not have been possible without your help.

I also thank the Dr. Lobner and Dr. Choi labs, and Dr.Khadijah Makky, as a significant amount of work was conducted with their help. Thank you for letting me use your equipment and your expertise. Thank you to Dr. Choi for tutoring me in the knowledge of molecular techniques and brain anatomy, I have learned so much from you. Many thanks to Dr. Jon Resch for teaching me about qPCR; to Matt Hurley and Brian Maunze for your continual assistance that has greatly contributed to this work. Thanks to Dr. Lobner, Julie Hjelmhaug, and Dr.Rebecca Albano for answering my endless questions about cell cultures and for your jokes that kept me entertained.

Also, many thanks to our collaborators, Tamara Nelson, Dr. Qing-Song Liu and Dr. Aron Geurts and their labs at the Medical College of Wisconsin for offering their unique expertise; to Dr. Jayme McReynolds, Dr. Zimei Wang, and the whole community of the Biomedical Sciences Department and all others who have lent their hands along the way.

To all my friends and the Goetz family, thank you for making graduate school a little easier and all these years in Milwaukee a great experience.

Lastly, I would like to say I am so grateful to have a warm and supportive family always there for me. Thank you to my parents, Xiangrui Kong and Yanqin Ouyang, for all your efforts and sacrifices to raise four of us during those difficult years, and to make us first-generation college students. It was from your actions that I have learned one could be kind and strong. Thanks to my sisters, Yufeng Kong, Lingpin Kong, and Yumei Kong, for making life so colorful. Though you are all in China, you have always been the sources of my strongest support.

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LIST OF ABBREVIATIONS

AARE	amino acid response element
AC	adenylate cyclase
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)-propionic acid
ANOVA	analysis of variance
AP	action potential
ARE	antioxidant responsive element
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPP	conditioned place preference
CREB	cyclic-AMP response element binding protein
CSF	cerebral spinal fluid
CTB	cholera toxin subunit B
DIG	digoxigenin
EAAT	excitatory amino acid transporter
eGFP	enhanced green fluorescent protein
eIF2	eukaryotic initiation factor 2
Epac	exchange factor directly activated by cAMP
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FGF-2	fibroblast growth factor 2
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
GLT-1	glial glutamate transporter -1
HBSS	Hanks ´balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IGF	insulin-like growth factor
iGluR	ionotropic glutamate receptor
IL	interleukin

IP3	inositol 1,4,5-trisphosphate
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mGluR	metabotropic glutamate receptor
MSK	mitogen and stress activated protein kinase
MSN	medium spiny neuron
NAc	nucleus accumbens
NaOH	sodium hydroxide
NMDA	N-methyl-D-Aspartate
Nrf2	eythroid 2-related factor 2
OCT	optimal cutting temperature
PACAP	pituitary adenylate cyclase-activating polypeptide
PCR	polymerase chain reaction
PFC	prefrontal cortex
PI3	phosphatidylinositol 3
PI3K	phosphatidylinositol 3 kinase
PIP2	phosphatidylinositol 4,5-bisphophate
PIP3	3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RT-qPCR	real-time quantitative
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
Slc7a11	solute carrier family 7, member 11
SN	substantia nigra
SSZ	sulfasalazine
Sxc	System x_c-
TBOA	DL-threo-β-benzyloxyaspartic acid
Trk	tyrosine kinase
VIP	vasoactive intestinal peptide
VP	ventral pallidum
VTA	ventral tegmental area
ZFN	Zing-finger nucleases

CHAPTER I

INTRODUCTION

General Introduction

Drug abuse and addiction have long been considered problems resulting from moral failures such as poor life decisions or a lack of willpower. Slowly, over the past several decades, scientific advances have progressively worked to break down this stigma. Today, addiction is defined by most medical associations as a chronic disease of the central nervous system (CNS). Similar to other diseases such as cancer and heart disease that are caused by a malfunction of the underlying physiological substrates, the disease of drug addiction is a result of drug-induced abnormal and persistent changes of the underlying organ, the brain.

One of the hallmarks of addiction is characterized by periods of heightened relapse susceptibility, even following protracted abstinence (Jaffe et al., 1989; Withers et al., 1995). As such, relapse is arguably the most important problem to address when developing treatments for addiction. Towards this, a better understanding of the biological basis of relapse becomes a top priority. Studies suggest that relapse originates from the persistent neuroadaptations at the molecular and cellular levels in the related brain circuit (Baker et al., 2003; Kalivas and Volkow, 2005; Nestler, 2005; Hyman et al., 2006; Koob and Volkow, 2010). Among these neuroadaptations, drug-induced changes in synaptic transmission due to aberrant glutamate signaling have been linked to behavior in virtually every preclinical model of drug seeking and have been found to correlate with the severity of drug craving in human substance abusers (Pierce et al., 1996; Breiter et al.,

1997; Volkow et al., 1999; Park et al., 2002; Baker et al., 2003; McFarland et al., 2003; McFarland et al., 2004; Schmidt et al., 2005; Volkow et al., 2005; Madayag et al., 2007; Kau et al., 2008; Ary et al., 2013).

It is becoming apparent that excitatory signaling is the product of a highly integrated activity of a network of glutamate release mechanisms, receptors, and transporters expressed by multiple cell types, especially neurons and astrocytes (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997; Danbolt, 2001; Schoepp, 2001; Baker et al., 2002; Baker et al., 2003; Pirttimaki et al., 2011; Bridges et al., 2012a). As a result of the complexity of this network, identifying approaches that can reverse cocaine-induced changes throughout the glutamate system has been challenging. This, at least in part, is due to the emphasis in most studies of a single cell type (e.g. astrocytes or neurons) while overlooking the fact that glutamate transmission results from the integrated actions of neurons and astrocytes. Therefore, understanding how these cells interact to achieve coordinated glutamate transmission is an essential goal for understanding the biological basis of drug relapse.

The main effort of this thesis is to explore neuronal regulation of a non-canonical glutamate transporter, system x_c- (Sxc) and how this novel form of neuron-astrocyte signaling contributes to synaptic transmission in the nucleus accumbens (NAc) efferents that control drug seeking behaviors. As a cystine-glutamate antiporter that is mainly located on astrocytes, Sxc plays an important role in neuronal glutamate signaling and has been repeatedly demonstrated to be involved in cocaine addiction (Baker et al., 2003; Madayag et al., 2007; Moussawi et al., 2009; Amen et al., 2011; Moussawi et al., 2011; Schmaal et al., 2012). In these studies, I focus on the regulation of Sxc by a neuropeptide,

pituitary adenylate cyclase-activating polypeptide (PACAP), which is exclusively expressed by neurons in the CNS (Figiel and Engele, 2000; Zhang et al., 2014), yet has been repeatedly demonstrated to regulate astrocytic functions, including glutamate transmission (Goursaud et al., 2008; Resch et al., 2014b). Therefore, the intersection of PACAP and Sxc on the regulation and coordination of neuronal and astrocytic glutamate systems presents itself as a unique opportunity to better understand this important and complex excitatory signaling network and how its disruption by drugs of abuse contributes to relapse.

Preclinical Models of Drug Seeking

The attempts to understand the biological basis of addiction have involved two primary approaches: brain imaging in humans and preclinical models of addiction that permit mechanistic-type studies. In this section, I will focus on the latter, although many key discoveries realized by preclinical studies have often been replicated in human studies (Grant et al., 1996; Breiter et al., 1997; Childress et al., 1999; Volkow et al., 2005).

Conditioned place preference

Addiction is a multi-faceted disorder. As such, various animal models have been developed to study the biological underpinnings of multiple components of addiction. For example, the conditioned place preference (CPP) can be used to study aspects of addiction related to drug context (Bardo and Bevins, 2000). This is because the CPP model is a form of classical conditioning, in which drug experience is associated with

certain contextual cues, demonstrated by a longer time spent in the environment associated with drug administration (Tzschentke, 2007). While useful in studying aspects of drug addiction related to the formation of contextual associations, this paradigm lacks face validity when compared to other models that enable self-administered drugs. Moreover, given that drug-induced plasticity can be intake-dependent, the CPP paradigm may lack construct validity since the amount of cocaine administered in this paradigm is relatively low.

Self-administration model

Drug self-administration model is often used to study factors that govern drug reinforcement and drug intake. In a typical self-administration paradigm as shown in Figure 1.1A, rats are trained to press an active lever that is equipped in the self-administration chamber, which triggers a computer interface controlling mechanical

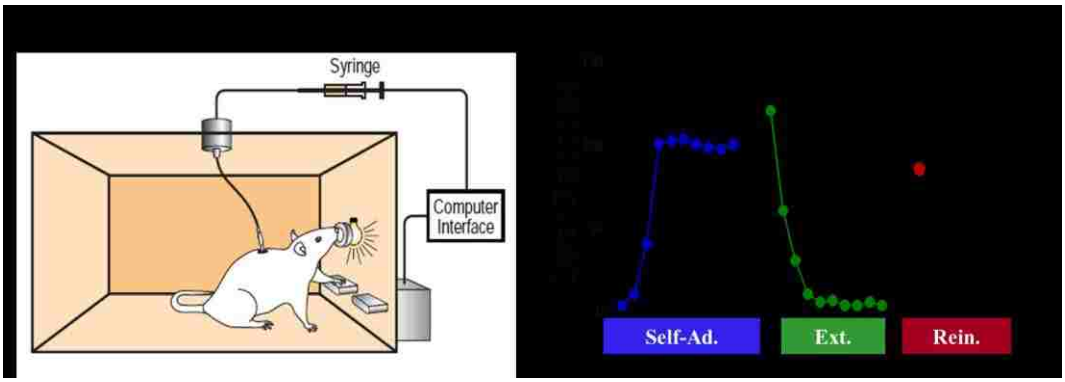

Figure 1.1. Self-administration and reinstatement models of drug seeking. A. Illustration of a typical self-administration chamber. B. Typical lever responses during drug self-administration (self-ad.), extinction training (ext.) and reinstatement test (rein.).

equipment to deliver an intravenous infusion of the drug through a catheter mounted on the back of the rats. As such, the animals self-administer drugs similarly to human subjects. Because drug self-administration is a form of operant conditioning, it permits

the study of factors that govern the reinforcing qualities of drugs of abuse. Further, the self-administration paradigm can be modified to study other aspects of addiction, including factors related to relapse, as described below.

Reinstatement model

The self-administration paradigm is often modified in order to study factors related to increases in non-reinforced drug seeking, which is used to model human relapse (Shaham et al., 2003; Lynch et al., 2010). Typically, the paradigm is extended such that subjects that have completed cocaine self-administration training, often to the point of having consumed enough cocaine to produce persistent drug-induced plasticity, undergo a series of manipulations designed to elicit non-reinforced operant responding (Shaham et al., 2003; Bossert et al., 2013). For example, many studies examine the factors enabling a priming dose of cocaine, which typically involves non-contingent administration to ensure the lack of reinforced responding, to produce significant levels of operant behavior. To facilitate the detection of increased behavior, extinction training can be used between the self-administration and reinstatement phases of the experiment (Figure 1.1B). Similar designs can also be used to test the capacity of stress, discrete drug-cues, and drug context to reinstate drug seeking (Lee et al., 2006; Crombag et al., 2008; Anker and Carroll, 2010; Mantsch et al., 2016). This paradigm has face validity in that the stimuli that cause relapse in humans can cause reinstatement of drug seeking in rodents (Gerber and Stretch, 1975; de Wit and Stewart, 1981; Self et al., 1996). In addition, the reinstatement model (incorporating self-administration) captures key features of human addiction, such as the reinforcing effect of the drugs, transition from recreational drug use to compulsive drug abuse, and resumption of drug intake after

prolonged periods of abstinence. Lastly, comparable data obtained from this animal model and human subjects supports the construct validity of this model. For example, studies with the reinstatement model demonstrated that activation of the prefrontal cortex (PFC) is required for the expression of drug-seeking behavior (Baker et al., 2003; Capriles et al., 2003; McFarland et al., 2003; McLaughlin and See, 2003), consistent with the results from clinical imaging studies (Grant et al., 1996; Breiter et al., 1997; Childress et al., 1999; Volkow et al., 2005). This indicates that there are shared biological substrates and mechanisms underlying relapse in both animals and human (Kalivas and McFarland, 2003).

With the aid of many neuroscience tools, including traditional techniques such as microdialysis (Baker et al., 2003; Madayag et al., 2010), microinjection (Blacktop et al., 2011; Graf et al., 2013), and also fast developing modern techniques such as chemogenetics (Scofield et al., 2015; Augur et al., 2016) and optogenetics (Stefanik and Kalivas, 2013; Stefanik et al., 2013), the reinstatement paradigm has proven to be a valuable preclinical model over the last few decades in elucidating the mechanisms underlying relapse. The value of this model is further supported by the discoveries of promising treatments for human drug addiction founded in part through the use of this model. For example, *N*-acetylcysteine was found to reduce cocaine-seeking in rodents and similarly to reduce drug craving in human subjects in clinical trials (Baker et al., 2003; LaRowe et al., 2007; Madayag et al., 2007; Amen et al., 2011; Moussawi et al., 2011; Reichel et al., 2011).

The Motive Circuit

The above paradigms have been widely used over the past 30+ years to specifically unmask the neuroanatomical and neurochemical systems underlying addiction. One of the major findings is that the rewarding properties of drugs of abuse and natural rewards such as food and sex share the same neural substrates that mediate motivated adaptive behaviors. Much of this work has led to the establishment of the motive circuit (Figure 1.2) as a key circuit involved in multiple components of drug addiction. Of particular importance, many findings over the past 25 years have supported

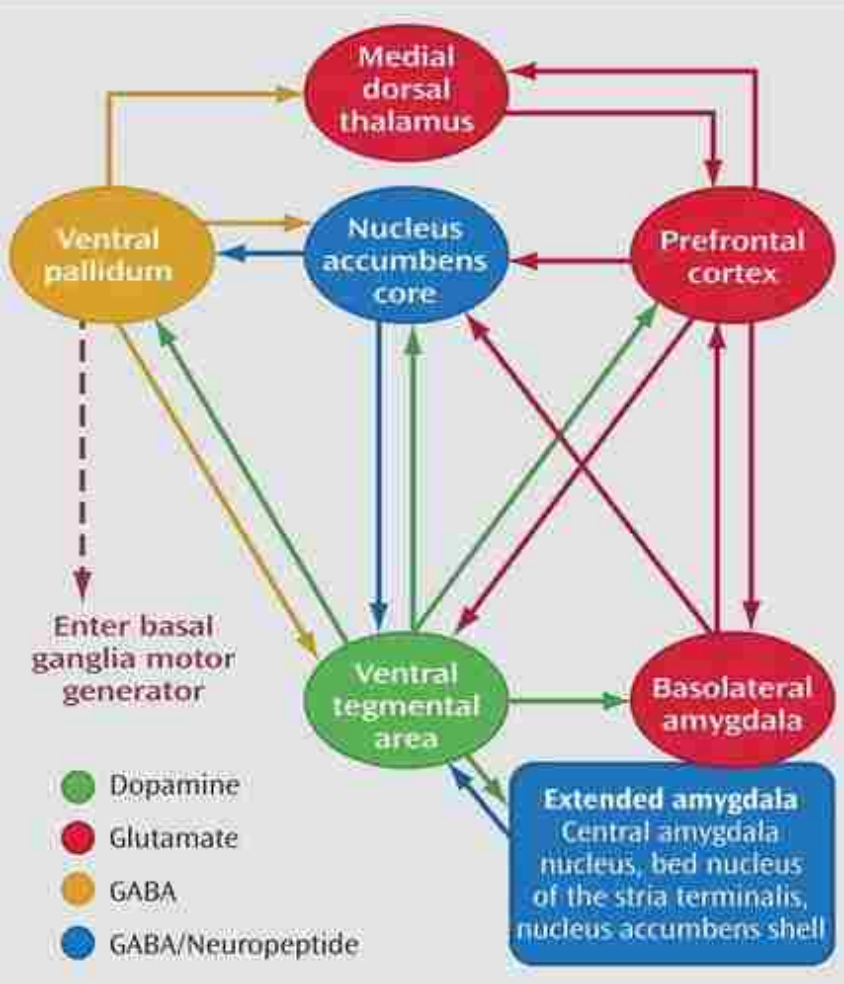

Figure 1.2. The motive circuit. Illustration of the interconnected brain regions and nuclei of the motive circuit. Adapted from (Kalivas and Volkow, 2005)

a central hypothesis in addiction that relapse and compulsive drug seeking arise from persistent neuroadaptations at molecular and cellular levels in the motive circuitry (Nestler, 2001; Kalivas and Volkow, 2005; Nestler, 2005; Kalivas, 2009).

The motive circuit, which is characterized by a collective of interconnected brain areas, including mainly the PFC, the amygdala, the hippocampus, the NAc, and the ventral tegmental area (VTA), is a circuit of neural networks that controls goal-directed behaviors.

The PFC, especially the medial portion (mPFC), is the executive center of the brain that is critical for decision making (Bechara et al., 2000; Rushworth et al., 2011; Euston et al., 2012). This function in part attributes to its anatomical position of receiving extensive projections directly from other cortical, subcortical and limbic regions, and therefore its capability of associating retrieved memory, sensory and emotional inputs (Miller and Cohen, 2001). As such, inputs from these cortical structures as an important component of the motive circuit enable executive function to contribute to the expression of goal-directed behaviors.

The amygdala is a forebrain limbic structure that is critical for the formation of emotional memory and thus conditioned learning (Rogan et al., 1997). In brief, it associates the motivationally relevant experiences, either rewarding or aversive, with the environmental stimuli, which subsequently predict the relevant events (Belova et al., 2007). As such, the amygdala, specifically, the basolateral amygdala has been heavily implicated in cue-induced drug-seeking behavior (See et al., 2001; Alleweireldt et al., 2002; Crombag et al., 2002). Therefore, inputs from the amygdala should be viewed as

enabling conditioned learning and/or emotional memory to also contribute to the expression of goal-directed behaviors.

The hippocampus, which is also part of the limbic cortex, is located in the temporal lobe of the brain and is a structure critical for the formation of contextual and declarative memories (Eichenbaum, 2000, 2004). Together with amygdala, these structures form and preserve detailed contextual memories of experiences, for example, those associated with drug taking (Kelley, 2004; Robbins et al., 2008). Hence, inputs from these structures enable contextual memories to contribute to the expression of goal-directed behaviors.

Midbrain nuclei, including the VTA, project throughout the motive circuit and are thought to release neurotransmitters, which collectively encode multiple aspects of motivated behavior (Adinoff, 2004; Alcaro et al., 2007). While these projections are primarily studied for their dopaminergic inputs, which have been long recognized to be strong modulators of motivated behavior (Kauer and Malenka, 2007; Gerfen and Surmeier, 2011), additional neurotransmitters include glutamate, gamma-aminobutyric acid (GABA), and co-neurotransmitters such as opioid peptides (Chuhma et al., 2009; Jarjour et al., 2009; Stuber et al., 2010; Tecuapetla et al., 2010; Tritsch et al., 2012). While much work is needed to parse out the components of motivated behavior encoded by each of these key neurotransmitters, it is clear that these inputs to the motive circuit enable goal-directed behavior to be a product of incentive salience, motivation, and reward.

Among all these structures in the motive circuit, this dissertation focuses on the NAc. The NAc is part of the ventral striatum and is located in the basal forebrain. It is a

central structure in the expression of goal-directed behaviors, in part because it connects the limbic subcircuit to the motor subcircuit. Specifically, it integrates inputs from the PFC, amygdala, hippocampus, and midbrain nuclei (Morgane et al., 2005). In turn, it then projects to the motor nuclei, ventral pallidum (VP) and the substantia nigra (SN). As such, the NAc is anatomically positioned as a gateway to integrate limbic information and lead to the expression of behaviors through recruitment of the motor system (Groenewegen et al., 1996; Yin and Knowlton, 2006).

Projections from the PFC to the NAc core have been shown to be involved in multiple forms of drug seeking (Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001; McFarland and Kalivas, 2001; Kalivas and Volkow, 2005). This glutamatergic projection and the NAc efferents to the motor system, simplified as the corticostriatal pathway, have been described to be the final common pathway for generating reinstatement behavior (Kalivas and Volkow, 2005). This is supported by its obligatory involvement in the initiation of drug seeking induced by all the three common triggers, i.e. cues, stress and drug (Cornish and Kalivas, 2000; McFarland and Kalivas, 2001; Park et al., 2002; Capriles et al., 2003; McFarland et al., 2003; McLaughlin and See, 2003; McFarland et al., 2004).

As introduced above, the development of addiction involves both the limbic and motor units of the corticostriatal pathway with the NAc serving as the gateway connecting the two (Kalivas, 2009). While the limbic subcircuit is critical for the development and establishment of addiction, such as the expression of drug seeking behavior, it also retains the power for altering well-established behaviors in order to adapt to constantly changing environment, in other words, generating new adaptive behaviors

(Barnes et al., 2005; Doya, 2008). For example, in the model of cocaine self-administration, when lever pressing reliably yields desired drug delivery, the influence of the limbic unit diminishes while the motor function gradually predominates in performing the task (Barnes et al., 2005; Yin and Knowlton, 2006). The same rules apply to the development of other habitual behaviors. However, when lever pressing fails to yield the expected drug delivery, such as in extinction training, the limbic subcircuit re-engages to re-evaluate environmental stimuli and reassign values, while the established motor pattern re-organizes concordantly. As such, a new adaptive behavior develops (Barnes et al., 2005; Doya, 2008).

These concepts indicate that drug-seeking behavior arises from the neuroadaptions that favor such established behavior, and/or that impair the capability of controlling or changing such behavior (Kalivas, 2009; Belin et al., 2013). In other words, drug reinstatement results from weakened limbic control and/or strengthened motor involvement. Consistently, extended drug intake promotes the progressive reliance on the motor systems, and at the same time the gradual disengagement of the limbic system (Ito et al., 2000; Ito et al., 2002; Porrino et al., 2004; Vanderschuren and Everitt, 2004; Vanderschuren et al., 2005; Pelloux et al., 2007).

As introduced above, the NAc is a gateway where limbic information gains its access for the motor expression. As such, dysfunction of this structure induced by repeated drug exposure might underlie the declining engagement of the limbic subcircuit. In fact, studies from the past two decades indicate that the imbalanced corticostriatal pathway arises at least in part from the malfunction of the glutamatergic signaling in the

NAc. This involves neuroadaptations in glutamate receptors, release and clearance mechanisms, for review see (Kalivas, 2009).

Glutamate Transmission in Addiction

Glutamate is the primary excitatory neurotransmitter in the brain (Curtis and Johnston, 1974; Choi, 1988; Harris and Kater, 1994; Meldrum, 2000; Franks et al., 2002; Javitt et al., 2011). Its transmission underlies virtually all brain functions, as supported by its involvement in numerous CNS disorders (Carlsson and Carlsson, 1990; Olney, 1990; Brown and Bal-Price, 2003; Hynd et al., 2004; Maragakis and Rothstein, 2004; Foster and Kemp, 2006; Niswender and Conn, 2010; Rondard and Pin, 2015).

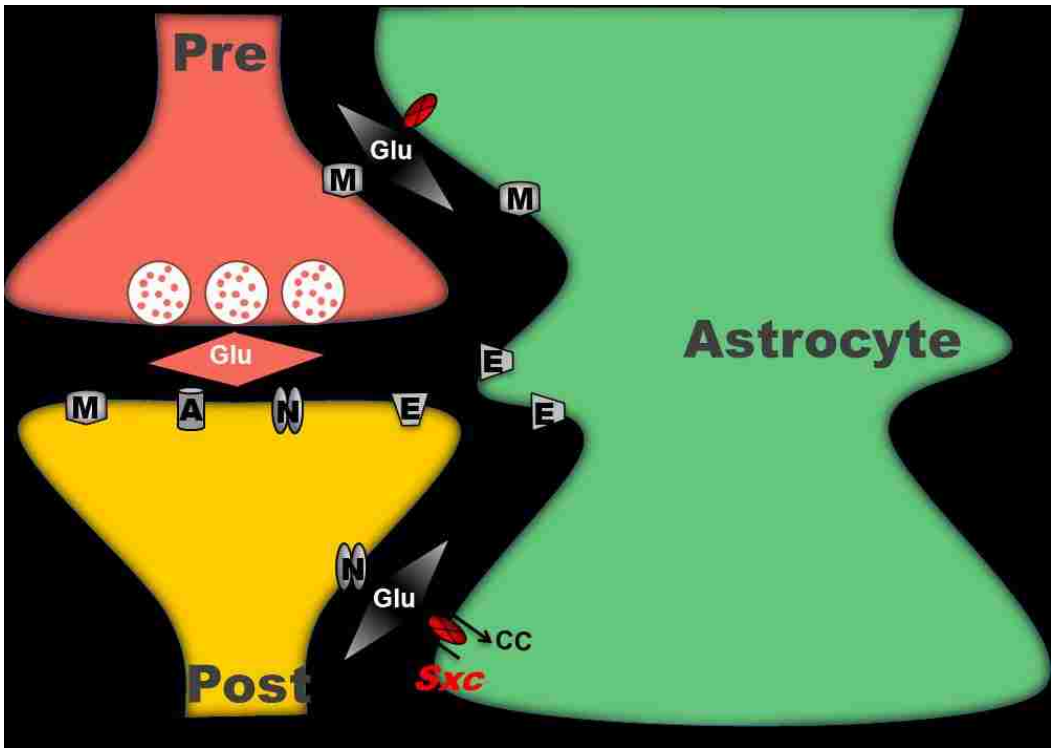

Figure 1.3. A typical glutamate synapse. Pre: presynaptic terminal; Post: postsynaptic terminal; Glu: glutamate; A: AMPA receptor; N: NMDA receptor; M: metabotropic glutamate receptor; E: excitatory amino acid transporter; CC: cystine; Sxc: system x_c- . Adapted and modified from (Bridges et al., 2012a).

With advances in neuroscience, it is gradually becoming apparent that glutamate neurotransmission is achieved by a complicated network that is formed by multiple cell types, including mainly neurons and astrocytes. Both of these cell types express glutamate receptors, transporters and release mechanisms (Figure 1.3).

Glutamate receptors

There are two types of glutamate receptors, ionotropic (iGluR) and metabotropic glutamate receptors (mGluR). iGluRs are ligand-gated ion channels that are formed by tetramers and mediate fast excitatory synaptic transmission (Dingledine et al., 1999; Platt, 2007; Traynelis et al., 2010). Based on their sequence differences and pharmacological ligand (agonist) selectivity, iGluRs are further divided into three subtypes, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), *N*-Methyl-D-aspartate receptor (NMDAR) and kainate receptor (Dingledine et al., 1999; Platt, 2007; Traynelis et al., 2010). These different receptor subtypes are distinctly distributed in the CNS and exhibit different channel properties determined by their subunit compositions and location (Dingledine et al., 1999; Platt, 2007; Traynelis et al., 2010). In this dissertation, I will focus on AMPARs and NMDARs, as they are the most studied iGluRs in addiction.

<u>AMPA receptors</u>

Most AMPARs are heterotetrameric, consisting of subunits GluA2 and either GluA1, GluA3, or GluA4, with a prototype of dimers of dimers (Nakagawa et al., 2005). Each of the four subunits harbors a binding site for glutamate. AMPARs are activated upon the occupation of as little as two binding sites by glutamate or agonists (Platt, 2007). The channel formed by the four subunits then opens to allow the influx of sodium

and potassium. The binding sites to glutamate are of low-affinity (EC50: 200-500 μM), and AMPARs deactivate fast due to the transient bound time of the glutamate that is caused by the rapid diffusion and dilution of the transmitter (Dingledine et al., 1999; Platt, 2007; Traynelis et al., 2010). Given that AMPARs are not voltage gated, this fast gating property renders AMPARs obligatory players in most of the fast excitatory neurotransmission (Zhang and Trussell, 1994; Edmonds et al., 1995; Palmer et al., 2005). Specifically, activation of AMPARs initiates depolarization that is essential for removing the magnesium blockers residing within NMDAR ion channels (Nowak et al., 1984; Dingledine et al., 1999). Thus, it facilitates the participation of NMDARs in generating the excitatory postsynaptic potential (EPSPs). As such, synaptic expression of AMPARs is critical in determining synaptic strength (Malinow and Malenka, 2002; Shepherd and Huganir, 2007).

Functional AMPARs are primarily located on postsynaptic terminals. The membrane expression of the receptors is a rather dynamic and highly amendable process (Anggono and Huganir, 2012; Niciu et al., 2012). It involves the expression of the receptors, the trafficking from the cell soma, and the lateral membrane diffusion from the extrasynaptic to the synaptic compartment (Groc and Choquet, 2006; Kennedy and Ehlers, 2006; Shepherd and Huganir, 2007; Newpher and Ehlers, 2008; Kapitein et al., 2010). The whole process is highly dependent on synaptic events, and in turn plays a key role in synaptic plasticity (Malinow and Malenka, 2002). For example, it has been well established that the integration of AMPARs to the synaptic membrane is critical in mediating long-term potentiation (LTP), a key mechanism that underlies learning and memory formation (Lynch, 2004). As such, AMPARs are broadly involved in

experience-induced neuroadaptations; disruption of AMPAR functions often negatively impact brain functions and has been demonstrated in various CNS disorders, including addiction.

AMPARs are enriched in the medium spiny neurons (MSNs) in the striatum (Martin et al., 1993; Bernard et al., 1997) where the NAc resides. Drug-induced AMPAR plasticity has been recurrently implicated in addiction studies. For example, repeated cocaine leads to increased surface expression of GluA1-containing AMPARs (GluA1-AMPARs) in the NAc, and consequently postsynaptic potentiation (Conrad et al., 2008; Moussawi et al., 2009). In addition, impairment of GluA1-AMPAR trafficking decreases cocaine induced reinstatement, suggesting that increased membrane expression of GluA1-AMPARs is critical for cocaine relapse (Anderson et al., 2008). Consistently, activating AMPARs with AMPA in the NAc reinstates cocaine-seeking behavior, while blocking AMPARs with receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) eliminates cocaine-induced reinstatement (Cornish and Kalivas, 2000).

Membrane expression of AMPARs is, to a certain degree, dependent on the phosphorylation of the receptor, a labile post-translational modification that is reversible and relatively dynamic (Wang et al., 2006; Mao et al., 2011). Phosphorylation sites exist on the C-terminals of each GluA subunit, which are intracellular domains that undergo protein-protein interaction and phosphorylation. There are four sites identified on the GluA1 intracellular domain, including serine 818 (S818), S831, S845 and threonine 840 (T840). Among these, T840 could be phosphorylated by p70S6 kinase and PKC (Delgado et al., 2007; Lee et al., 2007). Dephosphorylation of GluA1-T840 by NMDAR-mediated signaling has been implicated in long-term depression (LTD) (Delgado et al., 2007).

S845 is a substrate for PKA. Phosphorylation of S845 by PKA in response to cellular activity facilitates synaptic trafficking of the receptor, augments AMPAR-mediated currents, and is critical for LTP (Roche et al., 1996; Derkach et al., 1999; Banke et al., 2000; Esteban et al., 2003). Phosphorylation of S845 in striatal neurons has been extensively studied in addiction. It contributes to the psychostimulant (e.g. cocaine)-induced increase of synaptic AMPAR expression, and consequently the potentiation of synaptic strength (Price et al., 1999; Snyder et al., 2000; Chao et al., 2002; Mangiavacchi and Wolf, 2004; Swayze et al., 2004). Therefore, this mechanism is likely correlated with the neuroadaptations that underlie drug-seeking behavior.

NMDA receptors

Similar to AMPAR, NMDARs are also heterotetrameric. The receptor is composed of subunits from three classes, including GluN1, GluN2, and GluN3. Each NMDAR contains two GluN1 obligatory subunits, and two GluN2 and/or GluN3 subunits (Dingledine et al., 1999; Platt, 2007; Traynelis et al., 2010). Unlike AMPARs that are activated by glutamate or agonists, opening of the NMDAR ion channel requires the binding of both glycine/D-serine (co-agonists) and glutamate, with glycine to GluN1 and glutamate to the other two subunits (Kleckner and Dingledine, 1988; Chen et al., 2005; Wolosker, 2006). Additionally, full occupation of all four binding sites appears to be necessary for maximum activation of the receptor. However, activation of the receptor and the opening of the NMDAR channel does not assure the ion flux, as the channel is constitutively blocked by magnesium, which could be removed by the rapid depolarization of the membrane potential mediated by AMPARs (Nowak et al., 1984; Dingledine et al., 1999). Unblocked NMDAR channels allow the influx of sodium, and

calcium, and the outflow of potassium (Dingledine et al., 1999; Liu and Zhang, 2000; Cull-Candy et al., 2001; Paoletti and Neyton, 2007). The intracellular surge of calcium from NMDARs leads to activation of different calcium-dependent signaling pathways that are differentially involved in the induction of LTP and LTD (Shouval et al., 2002; Citri and Malenka, 2008; Luscher and Malenka, 2012).

In contrast to AMPARs, NMDARs demonstrate much higher affinity for glutamate. The affinity varies depending on the subunit composition and splicing variance of each receptor, yet the EC50 of NMDARs typically falls at micromolar or sub-micromolar level (McBain and Mayer, 1994; Meldrum, 2000). Unlike AMPARs, NMDARs activate and deactivate much slower (Edmonds et al., 1995; Wyllie et al., 1998), and thus mediate the slow component of the EPSP (Meldrum, 2000; Vargas-Caballero and Robinson, 2004). The slow kinetics of NMDARs allows a temporal summation of depolarization which generates a persistent excitation and thus is critical for neuronal firing (Herron et al., 1986; Daw et al., 1993; Larkum and Nevian, 2008; Polsky et al., 2009). Given that ion flux through NMDARs requires both the binding of glutamate that is released from the presynaptic terminal and the removal of channel blockers by postsynaptic depolarization, the slow kinetics of the receptor render NMDARs the capability to simultaneously detect both the pre- and post-synaptic activities that are necessary for the induction of LTP and LTD (Malenka and Nicoll, 1993; Luscher and Malenka, 2012). As such, NMDAR-mediated synaptic plasticity has been extensively studied in learning and memory formation (Tsien et al., 1996; Nakazawa et al., 2004).

NMDARs are enriched in the striatum and are predominantly GluN2A- and GluN2B-containing in this region; they are widely expressed by MSNs projecting to both the SN and the VP, and cholinergic interneurons (Landwehrmeyer et al., 1995; Chen et al., 1996; Dunah and Standaert, 2003). The concentrated expression of NMDARs in the striatum and their critical role in synaptic transmission and plasticity implicate that NMDARs are likely involved in addiction. In support, blocking NMDARs in the NAc induces reinstatement of drug seeking (Cornish and Kalivas, 2000; Famous et al., 2007). While not studied as extensively as AMPARs, NMDARs also demonstrate drug-induced plasticity. For example, cocaine self-administration alters the phosphorylation of striatal NMDARs in rats (Edwards et al., 2007). Such plasticity may in turn contribute to drug craving and relapse.

Metabotropic glutamate receptors

In comparison to iGluRs, which are ion channels that mediate fast synaptic transmission, mGluRs are G-protein coupled receptors that mediate their modulatory effects through activation of G-proteins and downstream second messenger systems (Pin et al., 2003). There are eight subtypes of mGluRs which are divided into three groups based on their amino acid compositions and signaling cascades (Niswender and Conn, 2010). Group I mGluRs (mGluR1 and 5) are coupled to Gαq, stimulation of which results in the calcium release from the endoplasmic reticulum (ER), as well as the activation of PKC, and thus related transduction cascades. Both group II (mGluR2 and 3) and group III mGluRs (mGluR4, 6, 7 and 8) are coupled to Gαi that negatively impact the activity of adenylate cyclase (AC). Therefore, activation of group II or group III mGluRs decreases

the production of cyclic adenosine monophosphate (cAMP), and consequently inhibits downstream signaling pathways including PKA-mediated cascades.

Both neurons and astrocytes express mGluRs. Neuronal mGluRs are located on presynaptic and extrasynaptic membranes. Activation of postsynaptic mGluRs has been shown to modulate ion channel activity (Haak et al., 1997; Benquet et al., 2002; Niswender and Conn, 2010), while presynaptic mGluR activation leads to reductions in presynaptic neurotransmitter release including glutamate and GABA (Schrader and Tasker, 1997; Mitchell and Silver, 2000; Pinheiro and Mulle, 2008). Such a mechanism is potentially involved in addiction. Specifically, repeated cocaine exposure leads to a persistent functional disruption of mGluR2/3 in the NAc (Xi et al., 2002). As presynaptic mGluR2/3 reduces the release probability of glutamate (Moran et al., 2005), this results in an augment of synaptic glutamate transmission and contributes to the reinstatement of cocaine seeking (McFarland et al., 2003; McFarland et al., 2004; Madayag et al., 2007; Miguens et al., 2008). Given that mGluR2/3 binds to glutamate with nanomolar high affinity (Muguruza et al., 2016) and is potentially activated by extrasynaptic glutamate released from Sxc (Xi et al., 2002; Losonczy et al., 2003; Moran et al., 2003; Grueter and Winder, 2005; Moran et al., 2005), cocaine-induced attenuation of Sxc in the NAc may exaggerate such consequences resulting from blunted mGluR2/3.

Glutamate clearance through EAATs

Extracellular glutamate needs to be maintained at a relatively low level, for at least two reasons. First, excessive stimulation of glutamate receptors by high concentrations of glutamate is toxic. For example, pathological surges of extracellular glutamate over-stimulate extrasynaptic glutamate receptors, which leads to cell death

(Hardingham et al., 2002; Ivanov et al., 2006; Leveille et al., 2008). Second, a relatively low level of extracellular glutamate is critical in limiting tonic activation of glutamate receptors (Danbolt, 2001). It has significant physiological meaning, because in such a manner, activation of glutamate receptors by activity/excitation-dependent glutamate becomes distinguishable from that induced by basal glutamate. As such, it provides appropriate signal-to-noise ratio (Danbolt, 2001). However, unlike many other neurotransmitters that can be enzymatically degraded, there is a lack of evidence for the existence of such an extracellular mechanism to metabolize glutamate. Therefore, a cellular uptake machinery is the only clearance mechanism that could rapidly eliminate the build-up of extracellular glutamate from constant synaptic release and other release mechanisms (Logan and Snyder, 1971; Balcar and Johnston, 1972; Danbolt, 2001). In fact, this function is mainly carried out by a family of cell-surface expressed proteins, excitatory amino acid transporters (EAATs).

EAATs are a family of Na+-dependent high-affinity glutamate transporters. They are symporters; 2-3 Na^+ and one proton are co-transported intracellularly with each molecule of glutamate, while a K+ is counter-transported to complete the circle and restore conformation of the transporter for the next circle (Rose et al., 2009). Five subtypes of EAATs (EAAT1-5) have been identified (O'Shea, 2002). EAAT1&2 are primarily expressed by astrocytes and EAAT 3-5 predominantly by neurons (Lehre et al., 1995; Anderson and Swanson, 2000; Pow and Barnett, 2000; Hu et al., 2003; Holmseth et al., 2012). Glial EAATs, EAAT1 and EAAT2 are believed to play the major role in extracellular glutamate clearance (Danbolt, 2001). EAAT1 is predominantly distributed in the neocortex and cerebellum and is the main transporter in the CNS during

development. EAAT2 is abundantly expressed in the forebrain; it occurs almost exclusively in astrocytes (Lehre et al., 1995), with particularly high densities in the astrocytic compartments that surround the synaptic cleft (Minelli et al., 2001). EAAT2 is the primary player in glutamate clearance, given that it accounts for as much as 90% of glutamate uptake in adult brain tissues (Haugeto et al., 1996; Tanaka et al., 1997; Danbolt, 2001). As such, disruption of glial glutamate transporter-1 (GLT1; rodent homologue of EAAT2) functions leads to toxic accumulation of extracellular glutamate (Rothstein et al., 1996; Tanaka et al., 1997).

Disruption of GLT1 has been repeatedly demonstrated in addiction studies. (Knackstedt et al., 2010; Fischer-Smith et al., 2012; Fischer et al., 2013; Reissner et al., 2014; Reissner et al., 2015). For example, cocaine self-administration reduces expression of GLT1 in the NAc (Knackstedt et al., 2010; Fischer-Smith et al., 2012; Trantham-Davidson et al., 2012). This down-regulation of GLT1 is likely involved in the drug relapse. In support, a number of studies demonstrated that restoration of GLT1 in animals with cocaine history prevented the reinstatement of drug-seeking behavior that is induced by cues or the drug, for review see (Roberts-Wolfe and Kalivas, 2015).

To date, studies of the regulation of EAAT2/GLT1 are still scarce. EAAT2/GLT1 undergoes post-translational alterations that are induced by several factors, including the substrate glutamate and arachidonic acid, resulting in a rapid activity change (Gegelashvili and Schousboe, 1998). Neurochemical signals, such as epidermal growth factor, influence EAAT2/GLT1 expression (Zelenaia et al., 2000; Su et al., 2003). Interestingly, several studies using *in vitro* cell cultures reveal that the expression of GLT1 in astrocytes is regulated by neurons or secretory neuronal factors including

PACAP, most likely through activating PKA (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al., 1998; Figiel and Engele, 2000). Neuronal regulation of GLT1 is further demonstrated *in vivo,* as the expression of this transporter is regulated by inputs from presynaptic neurons (Yang et al., 2009).

Glutamate release through Sxc

System x_c- (Sxc) is a sodium-independent, chloride-dependent cystine-glutamate antiporter (Bannai and Kitamura, 1980, 1981; Sato et al., 1999). It belongs to the heteromeric amino acid transporter family that are glycoprotein associated amino acid exchangers (Broer and Wagner, 2002; Verrey et al., 2004; Palacin et al., 2005). Functional Sxc, expressed on plasma membrane, is a heterodimer that consists of a heavy chain 4F2 (corresponding gene SLC3A2) and a light chain xCT (corresponding gene SLC7A11) (Sato et al., 1999). The two subunits are covalently linked through a disulfide bond (Bridges et al., 2012b). While the 4F2 heavy chain is shared by multiple amino acid transport systems and contributes to membrane expression of Sxc, the light chain xCT is unique to Sxc and determines its substrate specificities to cystine and glutamate (Sato et al., 1999; Sato et al., 2000; Bridges et al., 2001). By driving the release of glutamate into the extracellular space, Sxc transports cystine molecules into the cytoplasm (Figure 1.3) at a 1:1 ratio (Sato et al., 1999). As such, Sxc serves as a non-vesicular glutamate releasing mechanism.

Functions of Sxc

Both cystine and glutamate play important roles in maintaining normal functions of the CNS. Intracellular transport of cystine through Sxc is a rate limiting step for the

synthesis of glutathione (Deneke and Fanburg, 1989; Kato et al., 1992; O'Connor et al., 1995), a major antioxidant in the CNS. It is synthesized mostly in astrocytes (Raps et al., 1989; Trenga et al., 1991), yet yields its protection against oxidative stress for not only astrocytes, but also neurons (O'Connor et al., 1995; Shih et al., 2003; He et al., 2015). Glutamate release from Sxc, on the other hand, is critical for maintaining extracellular glutamate level/glutamate homeostasis. As introduced above, glutamate and glutamate receptors exist both within and outside of a synapse. Glutamate that is released from presynaptic terminals into the synaptic cleft contributes to the synaptic pool of glutamate. In contrast, extrasynaptic glutamate originates from mainly astrocytes through multiple mechanisms, including calcium-dependent vesicular release (Araque et al., 2000; Bezzi et al., 2004; Montana et al., 2004; Malarkey and Parpura, 2008), hemi-channels (Ye et al., 2003), and Sxc (Murphy et al., 1990; Warr et al., 1999; Baker et al., 2002). Among these, non-vesicular release from Sxc has been implicated to be the major source of basal glutamate in brain regions including the NAc (Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011).

Extrasynaptic glutamate is important in shaping synaptic transmission and plasticity by activating extrasynaptic receptors. For example, activation of presynaptic autoreceptor mGluR2/3 negatively regulates synaptic glutamate release. Such an inhibitory effect is facilitated by Sxc function, which provides extracellular glutamate for the activation of mGluR2/3 (Manzoni et al., 1997; Moran et al., 2005; Moussawi and Kalivas, 2010). Extrasynaptic NMDARs facilitate the induction of LTD (Liu et al., 2013), while excessive activation of extrasynaptic NMDARs is involved in the inhibition of LTP (Katagiri et al., 2001; Izumi et al., 2008; Li et al., 2011). Interestingly, extrasynaptic

NMDARs cluster at the perisynaptic domain and dendritic shaft (Petralia et al., 2010), where they are in close proximity to processes from neighboring cells including astrocytes (Tao-Cheng et al., 2007), and thus are likely to be activated by astrocyte-released glutamate (Angulo et al., 2004; Fellin et al., 2004; Le Meur et al., 2007; Shigetomi et al., 2008; Nie et al., 2010). In support, it has been shown that activation of extrasynaptic NMDARs requires glutamate release from glia (Bezzi et al., 2004; Fellin et al., 2004; Bergersen and Gundersen, 2009; Hamilton and Attwell, 2010; Bergersen et al., 2012). However, the direct link between Sxc and extrasynaptic NMDAR activation remains unclear. Sxc originated glutamate has been shown to attenuate postsynaptic clustering of glutamate receptors (Augustin et al., 2007). It also facilitates postsynaptic removal of AMPAR, resulting in the attenuation of synaptic strength (Williams and Featherstone, 2014). Consistently, loss of functional Sxc in mice leads to decreased levels of basal glutamate, and disruption of LTP, working memory and long-term memory (De Bundel et al., 2011; Li et al., 2012).

Therefore, dysregulation/dysfunction of Sxc has been shown to cause disrupted glutamate homeostasis and inability in coping with oxidative stress, and is implicated in various neuropsychiatric and neurodegenerative diseases, including addiction, schizophrenia, amyotrophic lateral sclerosis and Parkinson's disease, for review see (Bridges et al., 2012a; Lewerenz et al., 2013). With the rodent model of drug seeking, it has been demonstrated that repeated cocaine exposure leads to a significant reduction in basal glutamate and disrupted glutamate transmission in the NAc, resulting from decreased function of Sxc, which consequently lead to the reinstatement of drug seeking (Baker et al., 2003; Madayag et al., 2007; Kau et al., 2008; Knackstedt et al., 2010). In

support, restoration of NAc Sxc activity by ceftriaxone or prodrug *N*-acetylcysteine normalizes the basal level of glutamate, and significantly decreases the reinstatement of cocaine-seeking behavior (Madayag et al., 2007; Kau et al., 2008; Knackstedt et al., 2010; Trantham-Davidson et al., 2012).

Regulation of Sxc

Regulation of Sxc has been shown at the expression and phosphorylation levels. Consistent with its role in antioxidant defense, Sxc activity is upregulated in response to oxidative stress (Kim et al., 2001; Dun et al., 2006; Mysona et al., 2009). This upregulation is likely mediated by the nuclear factor erythroid 2-related factor 2 (Nrf2), which could be induced by oxidative insults and increases xCT transcription through interacting with the Nrf2-antioxidant responsive element (ARE) sequences on the SLC7A11 promoter (Itoh et al., 1997; Sasaki et al., 2002; Itoh et al., 2004; Shih et al., 2006). Such a pathway also underlies ceftriaxone-induced upregulation of xCT in astrocytes (Lewerenz and Maher, 2009). Another transcription factor identified is the eukaryotic initiation factor-2-activating transcription factor 4 (ATF4). By binding to the ATF4-amino acid response elements (AAREs) on the SLC7A11 promoter, it increases the transcription of xCT (Sato et al., 2004; Lewerenz and Maher, 2009; Lewerenz et al., 2012b). In addition to its involvement in coping with oxidative stress, this pathway mediates the upregulation of Sxc by insulin-like growth factor-1 (IGF-1), and may be induced in response to neuronal activity (Lewerenz et al., 2014; Yang and Yee, 2014). Fibroblast growth factor-2 (FGF-2) has also been implicated in increasing Sxc activity and xCT expression by recruiting both PI3K/Akt and MEK/ERK cascades (Liu et al., 2012; Liu et al., 2014a).

Studies suggest that Sxc activity is also influenced by post-translational modification, such as phosphorylation. For example, xCT harbors multiple phosphorylation sites, including those for PKA (Baker et al., 2002; McClatchy et al., 2016). Besides, the phosphorylation states are subjected to regulation and have been shown to influence the activity of Sxc (McClatchy et al., 2016). It has also been demonstrated that inhibiting cAMP and hence PKA activity rapidly decreases Sxc activity in rat striatal tissue slices (Baker et al., 2002). Interestingly, cAMP mediated signaling also appears to increase the expression of Sxc (Gochenauer and Robinson, 2001). In spite of the important roles of Sxc in brain functions such as its involvement in motivated behavior, the endogenous mechanism that mediates the regulation of Sxc is still not clear.

Neuron-Astrocyte Interactions

As introduced above, both neurons and astrocytes possess glutamate receptors, release and uptake mechanisms, which form a glutamate network that mediates the primary excitatory neurotransmission. A large body of studies suggests that these two cell types must coordinate to reach organized glutamate signaling.

Astrocytes

It has been frequently stated in the literature, especially in glia studies, that glia far outnumber neurons (commonly used ratio, 10:1) in the human or non-human primate brains. However, there is no direct evidence that supports such a remark. In fact, the most compelling study quantifying glia and neurons suggests a 1:1 ratio in the human brain

(Herculano-Houzel, 2009) and this ratio varies depending on brain regions. Regardless, glia compose no less than half of the human brain, and the ratio is probably one of the lesser questions, since prevalence does not equal significance. What is important, is that there is accumulating evidence suggesting that glial participation is critical for every major aspect of brain functions, for review see (Barres, 2008; Oliveira et al., 2015).

There are two major classes of glia in the CNS, macroglia (astrocytes and oligodendrocytes) and microglia. Microglia function as macrophages in the CNS, while the major role of oligodendrocytes is to provide axonal myelination. In this dissertation, I will focus on astrocytes.

Astrocytes are the major type of macroglia in the CNS. Anatomically, astrocytes are grouped into two main groups, protoplasmic astrocytes that are mainly distributed in the grey matter, and fibrillary astrocytes mainly in the white matter (Molofsky et al., 2012). These two groups of astrocytes demonstrate different morphologies, antigenic phenotypes/biochemical markers and likely different functions. Astrocytes are often times identified with the expression of glial fibrillary acidic protein (GFAP), the reason for this protein to be routinely used as an astrocyte-specific marker. However, it is necessary to mention that not every astrocyte expresses GFAP, and GFAP-expressing cells may be precursors for neurons (Alvarez-Buylla et al., 2001; Bushong et al., 2002).

Neuron-astrocyte interactions

Metabolism and homeostasis

Astrocytes typically harbor numerous membranous processes that give its star-like shape, as implicated in the name. Through these end-feet, astrocytes are in close

contact with both vasculature and synapses (Grosche et al., 1999; Ventura and Harris, 1999; Abbott et al., 2006). Such anatomical positions contribute to the central role of astrocytes in regulating brain microcirculation (Anderson and Nedergaard, 2003; Zonta et al., 2003; Takano et al., 2006). Specifically, astrocytes mediate vasodilation to boost local blood supply in response to increased synaptic/neural activity of a specific brain area. The metabolic supporting roles of astrocytes also includes serving as a glycogen reservoir (Brown et al., 2004; Brown and Ransom, 2007; Vilchez et al., 2007). Glycogen is converted to lactose and glucose, which are exported to neurons and used as fuel (Dringen et al., 1993; Ghosh et al., 2005). Interestingly, a recent study shows that instead of providing glucose to neurons in response to neuronal activity, astrocytes actively influence neuronal activity by gating the energy source to neurons (Garcia-Caceres et al., 2016). Astrocytes are also critical in maintaining extracellular ion levels and neurotransmitter (e.g. glutamate) homeostasis.

Gliotransmission and synaptic transmission

One of the principle differences between neurons and astrocytes is that neurons possess dendrites and axons and are capable of generating action potentials and long distance signal transductions (Raps et al., 1989). In contrast, astrocytes are incapable of AP-mediated signal transmission due to the low density of sodium channels (Vijayaraghavan, 2009). This somewhat contributes to the long-standing stigma that astrocytes do not participate in neurotransmission. However, this point of view has been challenged by a growing body of evidence, which implicates the direct involvement and critical role of astrocytes in synaptic transmission.

Similar to neurons, astrocytes express various receptors for neurotransmitters, including AMPARs (Seifert et al., 1997b; Seifert et al., 1997a), NMDARs (Lalo et al., 2006; Verkhratsky and Kirchhoff, 2007), mGluRs (Shelton and McCarthy, 1999), and GABA receptors (Fraser et al., 1994; Fraser et al., 1995). Electron microscopy studies have indicated that a number of astrocyte receptors are in proximity of neurotransmitter releasing sites on synapse, suggesting that astrocytes respond to neuronal neurotransmitters (Aoki, 1992; Douyard et al., 2007). In support, NMDAR-mediated currents have been demonstrated in astrocytes in response to synaptic neurotransmitter release (Lalo et al., 2006).

Even though astrocytes are not capable of generating action potentials, they demonstrate a special form of excitability and signal transduction. It is mediated by sophisticated calcium transmission that appears in the form of calcium waves and propagates through the cell volume (Yagodin et al., 1994; Fiacco et al., 2007). The calcium transients could be induced via activation of G-protein coupled receptors by various transmitters (e.g. glutamate) that are potentially released by neurons (Agulhon et al., 2008). For example, activation of mGluRs induces the Gαq mediated cascades and the production of inositol trisphosphates (IP3). IP3 binds to the IP3 receptors (IP3R) on the ER, which leads to calcium release from ER stores, and consequently, the intracellular calcium propagation along clusters of IP3R (Porter and McCarthy, 1996; Pasti et al., 1997; Perea and Araque, 2005; Fiacco et al., 2007; Weerth et al., 2007).

Astrocytes are capable of releasing several gliotransmitters, including glutamate, D-serine, adenosine triphosphate (ATP) and its metabolic byproduct adenosine, for review see (Haydon, 2001; Agulhon et al., 2008; Araque et al., 2014). All of these

molecules have corresponding receptors on the neurons. To date, it is well-established that activation of neuronal receptors by gliotransmitters is actively involved in synaptic transmission, plasticity and neuronal functions. For example, ATP released by astrocytes modulates synaptic release of neurotransmitters (e.g. glutamate, GABA and dopamine), as well as postsynaptic responses. D-serine acts as a co-agonist for NMDARs by interacting with the glycine-binding site of GluN1. Also, astrocyte glutamate, including that released from Sxc, is involved in various synaptic functions by activating a spectrum of presynaptic and extrasynaptic glutamate receptors, for review see (Agulhon et al., 2008; Araque et al., 2014).

To date, the releasing mechanism of these neuroactive substances is still not fully understood. A large body of evidence obtained from *in vitro* and *in situ* studies implicates that gliotransmitter release is dependent on the calcium transient-mediated exocytosis that is potentially induced by neuronal activity, for review see (Araque et al., 2014). Among these, vesicular exocytosis of glutamate has received considerable attention (Bezzi et al., 2004; Montana et al., 2004; Zhang et al., 2004; Crippa et al., 2006). However, the existence of this mechanism has been challenged by a study demonstrating the lack of known machinery that is required for vesicular glutamate release in mature mouse astrocytes (Barres, 2008; Cahoy et al., 2008). Therefore, whether or not vesicular glutamate release occurs *in vivo* remains a topic of debate (Volterra and Meldolesi, 2005; Barres, 2008; Hamilton and Attwell, 2010; Parpura and Zorec, 2010). Notably, among the glutamate releasing mechanisms expressed by astrocytes, Sxc is not frequently considered (Haydon, 2001; Araque et al., 2014).

Synaptogenesis and synaptic elimination

The impact of neuron-astrocyte interaction is not limited to its influence on metabolism and synaptic functions. It also appears to be critical for brain development and functions. For example, synaptogenesis is not simply controlled by pre and postsynaptic neurons (Christopherson et al., 2005), but is dependent on the facilitation from astrocytes. In fact, astrocytes are involved in the formation, elimination, maturation and maintenance of synapses (Slezak and Pfrieger, 2003; Christopherson et al., 2005; Kucukdereli et al., 2011; Chung et al., 2013; Clarke and Barres, 2013; Chung et al., 2015). In support, it has been recently postulated that astrocytes are involved in neurodevelopmental diseases such as autism and schizophrenia, both of which demonstrate abnormal synaptogenesis or synaptic elimination (Clarke and Barres, 2013; Sloan and Barres, 2014; Petrelli et al., 2016).

Neuronal regulation of astrocytes

Conversely, neurons appear to be critically involved in the differentiation and maturation of astrocytes, for review see (Stipursky et al., 2011). For example, cortical neurons regulate the onset of cortical gliogenesis during early brain development (Barnabe-Heider et al., 2005). In addition, neurons promote the expression of astrocyte GFAP *in vitro* via soluble factors (Gomes et al., 1999; de Sampaio e Spohr et al., 2002). Interestingly, such a function is influenced by the origin of the astrocytes and also the developmental stage of the neurons. Similarly, as mentioned earlier, expression of GLT1 in astrocytes is significantly influenced by the presence of neurons or secretory neuronal factors including PACAP (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al.,

1998; Figiel and Engele, 2000). In addition, neurons influence molecular and functional phenotypes of local astrocytes *in vivo* (Farmer et al., 2016). Clearly, neuron-to-astrocyte communication plays a critical role in astrocyte functions. However, the underlying mechanisms are not clear and the related studies, especially those investigating the neuronal regulation of astrocytic machinery that is involved in astrocyte-to-neuron communication (e.g. Sxc), are still scarce.

PACAP

PACAP (pituitary adenylate cyclase-activating polypeptide) was first discovered in ovine hypothalamus and named by virtue of its high potency in activating adenylyl cyclase (AC) in pituitary cells (Miyata et al., 1989; Arimura, 2007). It was subsequently found that PACAP is widely expressed by both the CNS and peripheral tissues (Vaudry et al., 2009). PACAP expression has been found in many species, including human, mouse, rat, fish, sheep, chicken (Vaudry et al., 2009). Moreover, the biologically active portion of PACAP amino acid sequence is almost completely preserved during evolution, as its structure is almost identical in species from fish to mammals, suggesting that the biological functions of PACAP may be essential (Vaudry et al., 2009). There are two forms of PACAP, the originally discovered PACAP1-38 that contains 38 amino acids, and PACAP1-27 which is a C-terminally truncated version of PACAP1-38 (Miyata et al., 1990). PACAP1-27 demonstrates a similarly high potency in stimulating AC compared to PACAP1-38 (EC50 for both: 0.3nM) (Miyata et al., 1990). In the CNS, the predominant form is PACAP1-38 (Arimura et al., 1991; Dickson and Finlayson, 2009). It is enriched in the hypothalamus, yet is expressed in a number of brain regions, including the cerebral

cortex and the amygdala (Arimura et al., 1991; Ghatei et al., 1993; Masuo et al., 1993; Fukuhara et al., 1997).

Studies using *in vitro* neural cell cultures demonstrate that PACAP is expressed exclusively by neurons (Figiel and Engele, 2000; Jaworski, 2000). Such cell-type specific expression patterns are likely applicable *in vivo*, as PACAP mRNA is only detected in cortical neurons compared to other cell types (Zhang et al., 2014). Interestingly, in the cortex, PACAP appears to be preferably expressed by glutamatergic and primarily pyramidal neurons in layer V (Koves et al., 1994; Hannibal et al., 2000; Hannibal, 2002a, b; Stumm et al., 2007; Engelund et al., 2010; Hu et al., 2011). Co-stores of PACAP and glutamate in nerve terminals also suggest that PACAP is co-transmitted through glutamatergic synapses (Hannibal et al., 2000; Hannibal, 2002a; Engelund et al., 2010). In support, it appears that the localization of PACAP does not parallel with the distribution of PACAP receptors, implicating that PACAP may function as a neurotransmitter (Masuo et al., 1992; Masuo et al., 1993; Golombek and Rosenstein, 2010; Purrier et al., 2014).

PACAP receptors

Three receptors, PAC1R, VPAC1R and VPAC2R have been identified to mediate PACAP signaling. These receptors belong to group B G-protein coupled receptor family (Harmar et al., 1998; Harmar, 2001). As is a major characteristic of this family, all three receptor subtypes are positively coupled to Gαs (Harmar et al., 1998; Harmar, 2001). Therefore, activation of these receptors by PACAP leads to stimulation of AC and consequently, intracellular accumulation of cAMP (Harmar, 2001). While VPAC receptors are mainly coupled to Gαs, PAC1R is reportedly paired with more diverse

transduction systems such as the activation of Gαq and the downstream cascades including the induction of PKC and the elevation of intracellular calcium (Arimura, 1998; Vaudry et al., 2000; Zhou et al., 2001; Dickson and Finlayson, 2009; Vaudry et al., 2009). PAC1R mRNA undergoes extensive splicing, resulting in variances in the transmembrane domain, N-terminal and intracellular loops, and hence corresponding splicing variants that possess differential signaling properties, including selectivity in coupling to downstream pathways, agonist potencies and maximal effects in response to agonist binding, for review see (Dickson and Finlayson, 2009; Vaudry et al., 2009; Blechman and Levkowitz, 2013).

PACAP receptors are also targets of vasoactive intestinal peptide (VIP) (Vaudry et al., 2009). Interestingly, PACAP and VIP have high resemblance in their amino acid sequences and both belong to the secretin superfamily, a collection of revolutionarily related peptides that exert a variety of functions through activating G-protein coupled receptors (Dickson and Finlayson, 2009; Vaudry et al., 2009). Out of the three receptors, PAC1R exhibits selectivity for its ligand, with a much higher affinity for PACAP (Kd: 0.5 nM) compared to VIP (Kd: 500 nM) (Cauvin et al., 1990; Gottschall et al., 1990; Lam et al., 1990; Gottschall et al., 1991; Suda et al., 1992). In contrast, VPAC receptors demonstrate similar affinities (Kd: 1 nM) for both PACAP and VIP (Gottschall et al., 1990; Lam et al., 1990).

Expression of the three PACAP receptors in the CNS is regionally different and cell-type specific. For example, PAC1R, as the major binding site for PACAP (Dejda et al., 2011), is the dominant form of all three subtypes in the cortex, while both VPAC receptors demonstrate lower levels of mRNA expression (Zhang et al., 2014).

Interestingly, PAC1R mRNA is primarily expressed by cortical astrocytes compared to all the other cell types (Zhang et al., 2014), while VPAC2R mRNA is also more abundant in cortical astrocytes compared to neurons.

PACAP functions

PACAP is a pleiotropic peptide, namely it has many different physiological functions (Vaudry et al., 2009). In the CNS, PACAP acts as a neurohormone, neurotropic factor, and also a neurotransmitter, for review see (Vaudry et al., 2009). In this dissertation, I will focus on the impact of PACAP on astrocyte functions, synaptic transmission, and the possibility of PACAP as a neuronal factor regulating Sxc.

Regulation of astrocytes

Consistent with the abundant occurrence of PACAP receptors in astrocytes (Zhang et al., 2014), PACAP exerts a number of actions on these cells, including the regulation of astrocyte proliferation, differentiation, plasticity and release of neuroactive substances. For example, PACAP at pico-molar concentrations promotes the proliferation of cultured astrocytes by recruiting MEK/ERK pathway (Hashimoto et al., 2003). Similarly, PACAP signaling induces astrocytic differentiation of cortical neural precursors (Vallejo and Vallejo, 2002; Seo and Lee, 2016), and appears critical in neocortical astrocytogenesis (Zupan et al., 1998). PACAP incubation increases the number and length of astrocyte processes *in vitro*, namely provoking the stellation (Ikeda et al., 2003; Perez et al., 2005). PACAP promotes the expression and release of neurotropic factors from astrocytes, including ciliary neurotrophic factor (CNF), activity-dependent neurotrophic factor (ADNF), activity-dependent neuroprotective protein

(ADNP), glial cell derived neurotrophic factor (GDNF), interleukin (IL) 1 and 6, macrophage inflammatory protein (MIP) and neurotrophin-3 (Masmoudi-Kouki et al., 2007; Vaudry et al., 2009). These factors, in turn, induce neuronal proliferation and/or differentiation, and promote neuroprotection.

PACAP also regulates gliotransmission. For example, PACAP induces endozepine release from cultured astrocytes, which, in turn, regulates neuronal activity through multiple mechanisms, including interaction with $GABA_A$ receptors (Bormann, 1991; Masmoudi et al., 2003; Compere et al., 2004, 2005; Masmoudi-Kouki et al., 2006; Papadopoulos et al., 2006). Interestingly, the precursor for endozepine appears to be exclusively expressed by astrocytes (Guidotti et al., 1983; Tong et al., 1991; Alho et al., 1995; Burgi et al., 1999). As such, neurons appear to exert self- or neuron-to-neuron regulation through the releasable factor, PACAP, which mediates neuron-to-astrocyte communication for recruiting astrocyte machinery. PACAP also regulates astrocyte glutamate transporters, which is important in maintaining glutamate homeostasis. For example, PACAP mediates neuronal regulation of the expression and activity of astrocyte GLT1 and glutamate aspartate transporter (GLAST) (Figiel and Engele, 2000). Consistently, activation of VPAC2R rapidly increases GLAST mediated glutamate uptake (Goursaud et al., 2008).

Regulation of synaptic transmission and plasticity

PACAP also regulates synaptic transmission and plasticity mediated by glutamate receptors, including NMDARs, AMPARs and mGluRs. Potentiation of NMDAR-mediated synaptic activity by PACAP has been repeatedly demonstrated (Liu and Madsen, 1997; Wu and Dun, 1997; Harrington et al., 1999). This function likely involves

the post-translational modification of NMDARs. For example, PACAP promotes phosphorylation of GluN2B and enhances NMDAR-mediated currents in hippocampal neurons (Yaka et al., 2003; Macdonald et al., 2005). Similarly, PACAP-induced regulation of GluN2B phosphorylation is also demonstrated in the hypothalamic ventromedial nuclei, and is involved in feeding behavior (Resch et al., 2014a).

PACAP differentially regulates AMPAR-mediated synaptic transmission in a dose-dependent manner; the amplitude of AMPAR-mediated excitatory postsynaptic currents (EPSCs) in hippocampal slices increase with a lower concentration of PACAP (i.e., 0.5 nM), while decreases with a higher concentration (i.e., 10 nM) (Costa et al., 2009). In contrast, another group showed that a high concentration of PACAP (100 nM) induces and amplifies AMPAR-mediated intracellular calcium concentrations in rat suprachiasmatic nucleus (SCN) neurons, while a low concentration (100 pM) fails to do so (Kopp et al., 2001). Moreover, it has been shown that PACAP alters hippocampal synaptic strength (Kondo et al., 1997; Roberto and Brunelli, 2000; Roberto et al., 2001; Ciranna and Cavallaro, 2003) and reduces synaptic expression of GluA1 (Gardoni et al., 2012). This function is likely mediated by post-translational events. For example, phosphorylation of T840 on the GluA1 subunits of AMPARs enhances channel conductance of AMPARs (Jenkins et al., 2014), while PACAP decreases GluA1-T840 phosphorylation (Toda and Huganir, 2015). Interestingly, the dephosphorylation of GluA1-T840 subunit induced by PACAP appears to be mediated by NMDARs (Toda and Huganir, 2015). In support, NMDARs have been demonstrated to regulate the phosphorylation of this site (Delgado et al., 2007; Lee et al., 2007; Gray et al., 2014), and as introduced above, PACAP regulates the function of NMDARs.

Though still preliminary, studies indicate that PACAP modulates group I mGluR-mediated calcium currents in rat SCN neurons. For example, 100nM PACAP inhibits intracellular calcium increases mediated by activation of mGluR1/5 (Kopp et al., 2001). In another study, however, PACAP (200nM) appears to promote mGluR1/5-coupled calcium currents in SCN and hippocampal neurons (Kammermeier, 2008).

Integrating neuron-astrocyte glutamate transmission through Sxc: a possibility

The broad impact of PACAP on glutamate machinery raises a question of whether or not PACAP also regulates astrocyte Sxc. In fact, a recent study by (Resch et al., 2014b) shows that PACAP increases Sxc activity in glial cultures from mouse cortices. Given the converging actions (or potential actions) of PACAP and Sxc on NMDAR functions and AMPAR-mediated synaptic transmission, and the potential link between NMDARs and AMPARs, as discussed above (Figure 1.4), we speculate that PACAP may be a neuronal factor that regulates Sxc in rat astrocytes, through which to integrate glutamate transmission across neurons and astrocytes, and therefore to achieve the proper expression of behaviors.

Summary

Drug addiction is a chronic CNS disorder characterized by persistent relapse susceptibility, even after years of abstinence. This results at least in part from drug-induced aberrant glutamate signaling within the corticostriatal projections of the motive circuit, the substrate in the brain that mediates motivated behaviors. A challenge in revealing the glutamatergic basis of CNS pathology is that excitatory neurotransmission

likely relies on integrated activities between neurons and astrocytes, yet how these cells interact to form a coordinated glutamate network is largely unknown. Therefore, in these studies, we aim to investigate whether the neuronal PACAP and the astrocyte Sxc form a critical link that orchestrates neuron-astrocyte communication to achieve integrated glutamate transmission that underlies motivated behavior. Specifically, we hypothesize that PACAP mediates neuronal regulation of Sxc in astrocytes, which, in turn, releases glutamate to activate extrasynaptic NMDARs, through which to modulate AMPAR functions (Figure 1.4). Findings from these studies should further our understanding of the complex excitatory signaling network and how its disruption by drugs of abuse may contribute to relapse. Beyond this, our findings may have implications for understanding other CNS conditions, given the fundamental role of glutamate in mediating the excitatory signaling that contributes numerous brain functions.

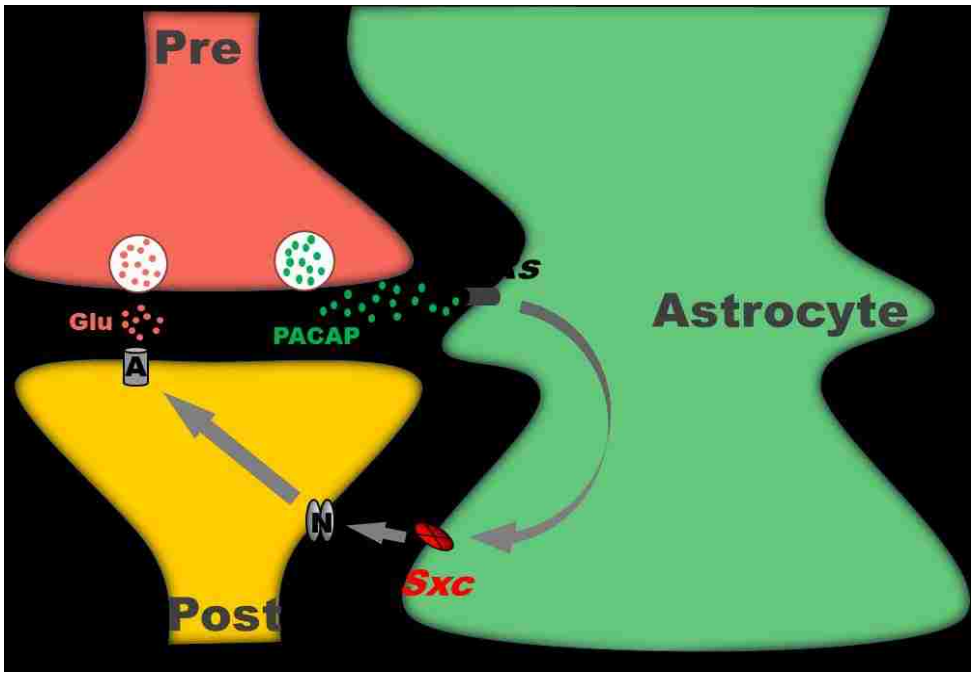

Figure 1.4. The hypothetical model. Schematic illustrates the hypothesized mechanistic model in this dissertation. Pre: presynaptic terminal; Post: postsynaptic terminal; Glu: glutamate; A: AMPA receptor; N: NMDA receptor; PRs: PACAP receptors; Sxc: system x_c-.

CHAPTER II

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ORCHESTRATES NEURONAL REGULATION OF THE ASTROCYTIC GLUTAMATE RELEASING MECHANISM SYSTEM X_C^-

Introduction

Glutamate is often described as the primary excitatory neurotransmitter in the brain, in part because it may be present in up to 80% of all synapses (Curtis and Johnston, 1974; Choi, 1988; Harris and Kater, 1994; Meldrum, 2000; Franks et al., 2002; Javitt et al., 2011). As such, altered excitatory neurotransmission likely contributes to most CNS diseases (Carlsson and Carlsson, 1990; Olney, 1990; Brown and Bal-Price, 2003; Hynd et al., 2004; Maragakis and Rothstein, 2004; Foster and Kemp, 2006; Niswender and Conn, 2010; Rondard and Pin, 2015). While traditional models of glutamate signaling depict release solely from presynaptic terminals and diffusion throughout the synaptic cleft to activate pre- and postsynaptic receptors, it is becoming increasingly evident that excitatory neurotransmission is achieved by an elaborate network expressed across multiple types of cells that regulate signaling within and outside of the synaptic cleft (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997; Jabaudon et al., 1999; Danbolt, 2001; Schoepp, 2001; Baker et al., 2002; Baker et al., 2003; Pirttimaki et al., 2011; Bridges et al., 2012a). In support, astrocytes and neurons express glutamate receptors, transporters, and release mechanisms, and astrocyte to neuron signaling has been shown to be a key determinant of synaptic transmission (Porter and McCarthy, 1996; Pasti et al., 1997; Araque et al., 1999; Fellin et al., 2004; Perea and Araque, 2005; Fellin et al., 2006a; Haydon and Carmignoto, 2006; Panatier et al., 2011; Santello et al.,

2012; Perez-Alvarez et al., 2014; Gomez-Gonzalo et al., 2015). Therefore, decoding the complex molecular and cellular regulation of glutamate could lead to novel opportunities to better understand and treat pathological excitatory signaling.

A critical gap in modeling excitatory signaling is how distinct components of the glutamate system expressed by neurons and astrocytes are coordinated. In these experiments, we tested the hypothesis that neurons regulate the activity of glutamate-related mechanisms expressed by astrocytes. To do this, we examined the neuronal regulation of system x_c- (Sxc), a non-canonical glutamate-release mechanism primarily expressed by astrocytes (Bannai and Kitamura, 1980, 1981; Sato et al., 1999; Pow, 2001; Zhang et al., 2014).

Sxc is a key component of glutamate signaling that is implicated in the pathology or treatment of multiple CNS diseases. It contributes to glutamate signaling by coupling the release of non-vesicular glutamate to the intracellular transport of cystine (Bannai and Kitamura, 1980, 1981; Sato et al., 1999). Manipulations that increase Sxc activity have been shown to a) influence multiple aspects of synaptic transmission and plasticity (Baker et al., 2002; Xi et al., 2002; Moran et al., 2005; Moussawi et al., 2009; Moussawi et al., 2011; Kupchik et al., 2012), b) normalize behavior in preclinical disease models (Baker et al., 2003; Madayag et al., 2007; Baker et al., 2008; Knackstedt et al., 2010; Alajaji et al., 2013; Lutgen et al., 2013), and c) exert therapeutic effects against multiple CNS diseases including drug addiction and schizophrenia (Berk et al., 2008; Amen et al., 2011; Lewerenz et al., 2013; Canavan et al., 2014; Verrico et al., 2014). Unfortunately, the regulation of Sxc is poorly understood.

In these studies, our primary objective was to evaluate the possibility that neurons regulate astrocyte Sxc. In addition, we tested the hypothesis that the pituitary adenylate cyclase-activating peptide (PACAP) is a neuronal factor capable of regulating astrocyte Sxc activity. To do this, we verified that PACAP is expressed by rat cortical neurons and not astrocytes, and that rat cortical astrocytes express PACAP receptors. We then found that application of PACAP to cortical astrocytes increased Sxc activity. Moreover, inhibition of PACAP signaling blocked neuron-induced upregulation of Sxc. Collectively, these data are consistent with the possibility that altered neuronal activity could give rise to pathological changes in astrocyte functions, including altered Sxc activity which may be present in numerous CNS disorders ranging from drug addiction to schizophrenia.

Material and Methods

Animals and materials: These experiments utilized cortical tissues obtained from Sprague Dawley rats (age was gestational day 15-16 or post-natal day 3-4; sex was undetermined; Envigo, Indianapolis, IN). Experimental procedures were approved by the Marquette University Institutional Animal Care and Use Committee. The primary materials included fetal bovine serum and horse serum (Atlanta Biologicals; Lawrenceville, GA), ^{14}C-cystine (PerkinElmer, Waltham, MA), rabbit anti-PACAP1-38 antiserum (final dilution 1:600; Peninsula Laboratories, San Carlos, CA), PACAP1-38 (California Peptide Research, Napa, CA), and PACAP6-38 (Anaspec, Fremont, CA). PACAP1-38 (PACAP) is the endogenous full-length peptide whereas PACAP6-38 (P6-38) is a truncated version of PACAP and inhibits PACAP receptors (Miyata et al., 1989; Robberecht et al., 1992; Arimura, 2007; Vaudry et al., 2009).

Cell culture procedures: Purified cortical astrocyte cultures were prepared from post-natal days 3-4 rat pups. In brief, cortical cells were disassociated and then suspended in Neurobasal A media (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, and 1% Glutamax (Thermo Fisher Scientific, Waltham, MA). Cells were initially grown in $25cm^2$ or $75cm^2$ cell culture flasks. Once confluent, cells were then subjected to prolonged, orbital shaking (250 rpm for 18 hours at 37^oC) which has been used to yield purified astrocyte cultures (McCarthy and de Vellis, 1980; Schildge et al., 2013). Purified astrocytes were then plated on 24-well plates, and refreshed with 70% new culture media every 2-3 days.

Purified cortical neuronal cultures were prepared from embryonic rat cortical tissue (gestational day 15 to 16) as previously described (Lobner, 2000). In brief, dissociated cells were suspended in Eagles' Minimal Essential Medium (MEM, Earle's salts, glutamine-free) supplemented with glutamine (2mM), glucose (21mM), horse serum (5%), and fetal bovine serum (5%). Cells were seeded on 24-well plates. Forty eight hours later, cytosine arabinoside (at a final concentration of 10 µM) was added to the culture media to inhibit glial reproduction (Dugan et al., 1995). Neurons were then grown for an additional 11-13 days.

Mixed Neuronal and Glial Cultures Procedure for preparing mixed cultures was identical to that of obtaining neuronal cultures (see above) except that cytosine arabinoside was not added to the culture media.

Physically Separated Astrocyte and Neuronal Cultures. Because the above mixed cultures also contained a limited number of non-astrocyte glial cells, and to determine whether neuron-astrocyte communication involves the release of a neuronal factor, we

utilized a “non-contact” co-culture system in which purified astrocytes were physically separated from purified neurons. Note, these cells are referenced in the manuscript as Astrocytes + NCM (neuronal conditioned media). In these experiments, purified astrocytes were obtained as described above. When astrocytes reached confluency after 13-16 days *in vitro* (DIV), neuronal cultures were seeded on removable inserts (Corning, Corning, NY) that had been placed into the wells containing astrocytes, such that both cell types were immersed in the media but were not in physical contact. Fourteen days later, downstream experiments to examine the effects of neurons on astrocytes were performed upon removal of the neuronal inserts. As a result, the DIV for these cells was 27-30.

All cell-growing surfaces in culture flasks and 24-well plates were pre-coated with poly-D-lysine (10mg/L) and laminin (0.4mg/L). All cell cultures were maintained in humidified 5% CO_2 incubators at 37^{o}C.

^{14}C-cystine uptake assay: The assessment of system x_c- (Sxc) activity is often achieved by measuring intracellular uptake of radiolabeled cystine since this is primarily dependent on Sxc (Liu et al., 2009; Liu et al., 2014a; Resch et al., 2014b; Albano et al., 2015). Note, we demonstrated that over 85% of ^{14}C-cystine uptake in our cells was blocked by the Sxc inhibitor sulfasalazine (SSZ; see Figure 2.3B). In contrast, extracellular glutamate is an insensitive indicator of Sxc since this is a product of numerous mechanisms (Swanson et al., 1997; Duan et al., 1999; Perego et al., 2000; Montana et al., 2004; Hires et al., 2008). Radiolabeled cystine uptake assays were performed as described previously with minor modifications (Liu et al., 2009). In brief, experiments were conducted in a bead bath at 37^{o}C. Cells were washed 3 times with

warm HEPES buffered saline solution, after which ^{14}C-cystine was added to the media for 20 min at a final concentration of 0.3μM. This concentration was used since it is similar to extracellular cystine concentrations in the brain (Baker et al., 2003). DL-threo-β-Hydroxyaspartic acid (TBOA; 10 μM) was added to the culture media to prevent ^{14}C-cystine uptake by sodium-dependent glutamate transporters. As described below, some of the experiments also involved the addition of the Sxc inhibitor SSZ (300 μM), PACAP, or P6-38. After incubating cells with ^{14}C-cystine, cells were washed 3 times with ice-cold HEPES buffered saline solution, and then solubilized with 1M NaOH solution. One aliquot of cell lysate was used for protein determination using a BCA protein assay, and another aliquot was used for scintillation counting to measure the level of ^{14}C-cystine uptake. ^{14}C-cystine content was normalized to protein concentration. Data are presented as CPM/μg of protein.

RNA extraction and cDNA construction: Total RNA was isolated from cell cultures with Trizol Reagent (Invitrogen; Carlsbad, CA) following the manufacture's protocol. DNase treatment was applied to all RNA samples to remove potential genomic DNA contamination with a DNA-free kit (Life Technologies, Carlsbad, CA). Assessment of RNA purity and quantity was performed on a NanoVue Plus Spectrophotometer (GE life sciences; Pittsburg, PA). cDNA was constructed from total RNA using the Reverse Transcription System (Promega, Madison, WI) with oligo(dT) primers following the manufacture's protocol.

Real-time quantitative PCR: Quantitative PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems; Carlsbad, CA) and PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersberg, MD). Relative quantification of

target gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). Primer sequences were as follows. xCT (catalytic subunit of Sxc) forward- 5' AGG GCA TAC TCC AGA ACA CG 3'; xCT reverse- 5' ATG CTC GTA CCC AAT TCA GC 3'; PACAP forward-5' AAC CCG CTG CAA GAC TTC TA 3'; PACAP reverse- 5' CTT TGC GGT AGG CTT CGT TA 3'; PAC1R forward- 5' TGC CTG TGG CTA TTG CTA TG 3'; PAC1R reverse- 5' TTT AGT CCC ATC AGG TCG TTG 3'; GAPDH forward- 5' CTC CCA TTC TTC CAC CTT TGA 3'; GAPDH reverse- 5' ATG TAG GCC ATG AGG TCC AC 3'. Primers were designed to be intron-spanning using the online primer design tool Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). A single product from amplification was confirmed by melt curve analysis. Amplification efficiency of all genes was determined to be approximately 95%.

Statistics: Statistical analyses were performed using SPSS Statistics (Version 19, IBM; Armonk, New York). Analysis of variance (ANOVA) was used when comparing data sets that included more than two groups. Bonferroni tests were used for subsequent *post hoc* analyses of significant effects involving more than two groups. Student's t tests were used when comparing results from only two groups. In all instances, statistical significance was designated as $p < 0.05$.

Experiment 1: Neuronal regulation of Sxc. The goal of this experiment was to test the hypothesis that a releasable neuronal factor regulates Sxc activity in astrocytes. To do this, we used two distinct approaches to co-express rat cortical neurons and astrocytes in culture. First, we measured ^{14}C-cystine uptake in rat mixed cortical cultures

(DIV 13-16). The advantage of this approach is that astrocytes are continuously grown in the presence of neurons. A disadvantage of this approach is that these cultures contain a limited number of microglia and oligodendrocytes. In addition, neurons physically contact astrocytes in these cultures. To address these two points, we also measured ^{14}C-cystine uptake by astrocytes that had been exposed to neuron-conditioned media. To do this, we first generated purified astrocyte cultures as described above. When astrocytes reached confluency (typically 13-16 DIV), neuronal cultures were seeded on removable inserts which were placed into the wells containing astrocytes, such that both cell types were co-cultured yet with no physical contact. Fourteen days later, the neuronal inserts were removed, and ^{14}C-cystine uptake was measured as described above. Note the 14-day period represents the time needed for the neurons to mature. The control cells in this experiment were purified astrocyte cultures in which the inserts lacked neurons. Tests using these cells occurred on DIV 27-30.

Experiment 2: Neuronal upregulation of Sxc requires PACAP receptor signaling. In an effort to begin to unmask the molecular basis of neuron-astrocyte signaling that regulates Sxc, we examined the impact of disrupting PACAP signaling on cystine uptake in rat cortical cultures. Our interest in PACAP stems from previous studies showing that stimulation of PACAP receptors increases the activity of Sxc and GLT1 (Figiel and Engele, 2000; Resch et al., 2014b), both of which are primarily expressed by astrocytes (Rothstein et al., 1994; Lehre et al., 1995; Rothstein et al., 1996; Torp et al., 1997; Pow, 2001; Zhang et al., 2014). To examine the involvement of PACAP, we modified the neuronal insertion design that was introduced in Experiment 1. In brief, media from cultured astrocytes only or astrocytes that were cocultured with

neurons (i.e., neuronal insertion) were applied to astrocytes (13-16 DIV) for 24 hours. PACAP antiserum was added to the media of some cultures during 24-hour incubation to neutralize the potential PACAP content (Figiel and Engele, 2000). Alternatively, we examined the impact of blocking PACAP receptors on cystine uptake. To do this, vehicle or 10 μM P6-38 (see Figiel and Engele, 2000 for concentration justification) was applied to rat cortical cultures (DIV 13-16) for 60 min, at which point ^{14}C-cystine uptake was measured as described above.

Experiment 3: PACAP upregulates Sxc on astrocytes. In this experiment, we tested the hypothesis that exogenous application of PACAP would significantly upregulate Sxc activity in purified astrocyte cultures. To test this, PACAP (0-100 nM) was applied to purified astrocyte cultures (DIV 13-16) for 24 hours. Afterwards, ^{14}C-cystine uptake was measured as described above. This experiment was also conducted in purified neuronal and mixed cortical cultures although the predicted outcome was less certain given the likely expression of endogenous PACAP in these cultures.

Experiment 4: Cellular distribution of PACAP and PAC1R in rat cortical cultures. To further examine the possibility that PACAP is a neuronal factor capable of regulating astrocytes, we examined the cellular expression patterns of PACAP and its primary receptor, PAC1R. We were specifically interested in determining whether PACAP was solely expressed in neurons and PAC1R in purified astrocytes. To do this, we used RT-qPCR to measure mRNA expression for PACAP and PAC1R in mixed cultures, purified astrocyte cultures, and purified neuronal cultures (DIV 13-16).

Experiment 5: Regulation of xCT mRNA by neurons and the neuronal factor PACAP. To better understand the observed upregulation of astrocyte Sxc by neurons or

PACAP, we measured xCT mRNA in purified astrocytes and mixed cultures, purified astrocytes ± neuronal-conditioned media, and purified astrocytes ± PACAP.

Results

Experiment 1: Neuronal regulation of Sxc. To determine whether neurons are capable of regulating astrocyte Sxc activity, we used two approaches to permit neuron-astrocyte signaling. First, astrocyte and neurons were co-cultured upon plating (mixed

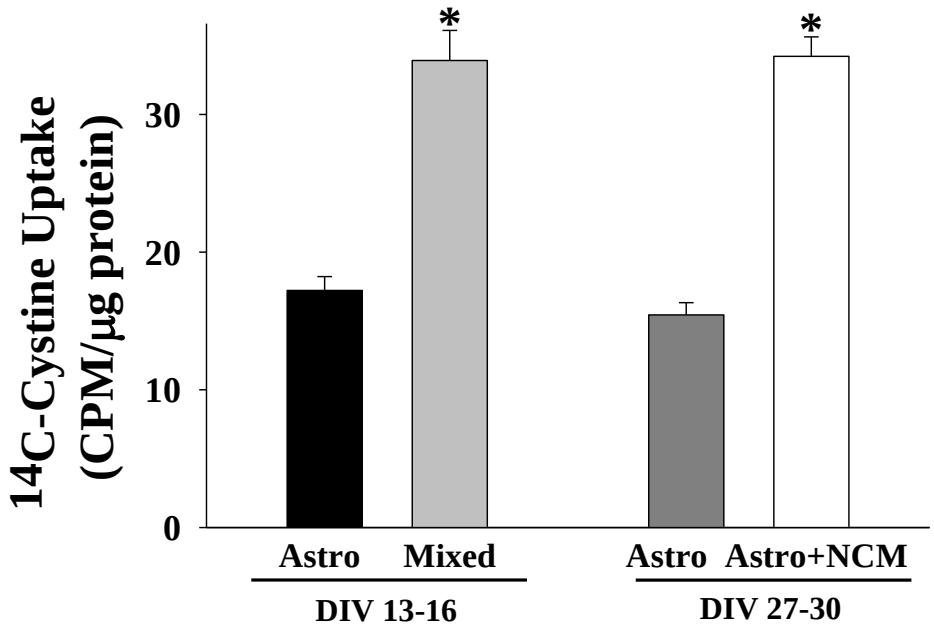

Figure 2.1. Neurons upregulate Sxc activity in astrocytes. Data depict the mean ± SEM levels of ^{14}C-cystine measured in rat cortical cells (N=8-12/condition) that had been cultured for 13-16 or 27-30 days *in vitro* (DIV). Astro, astrocytes; NCM, neuronal conditioned media. * represents a significant difference from corresponding purified astrocytes, $p < 0.001$.

cultures). This approach is designated in Figure 2.1 as DIV 13-16, which refers to the number of days the cells had been cultured. In addition, confluent, purified astrocyte cultures were exposed to neuronal conditioned media (NCM) by placing neurons grown onto an insert into the wells containing the astrocytes for 14 days (as described in the

methods for experiment 1). This approach is designated in Figure 2.1 as DIV 27-30. A univariate ANOVA with DIV and culture cellular composition (i.e., astrocytes only or mixed or astrocyte + NCM) as between subjects factors produced a main effect of cell composition [Figure 2.1; $F(1,36)=77.979$, $p < 0.001$] but not a main effect of DIV or an interaction between these variables. These findings illustrate that a releasable neuronal factor significantly upregulates Sxc activity on astrocytes (see Figure 2.1).

Experiment 2: Neuronal upregulation of Sxc requires PACAP receptor signaling. Previous studies have shown that PACAP increases the activity of Sxc and GLT1 (Figiel and Engele, 2000; Resch et al., 2014b), both of which are primarily expressed by astrocytes (Rothstein et al., 1994; Lehre et al., 1995; Rothstein et al., 1996; Torp et al., 1997; Pow, 2001; Zhang et al., 2014). In an effort to reveal the molecular basis of neuron-astrocyte signaling that regulates Sxc, we examined the impact of inhibiting PACAP signaling via neutralizing PACAP on the ^{14}C-cystine uptake in rat cortical cultures. Twenty-four hours later, Sxc activity was assessed with cystine uptake assay (Figure 2.2A). A univariate ANOVA with media origin (ACM vs NACM) and PACAP antiserum treatment (vehicle or 1:600 antiserum) as between-subjects variables yielded a significant interaction [Figure 2.2A; $F(1,30)=10.577$, $p < 0.01$].

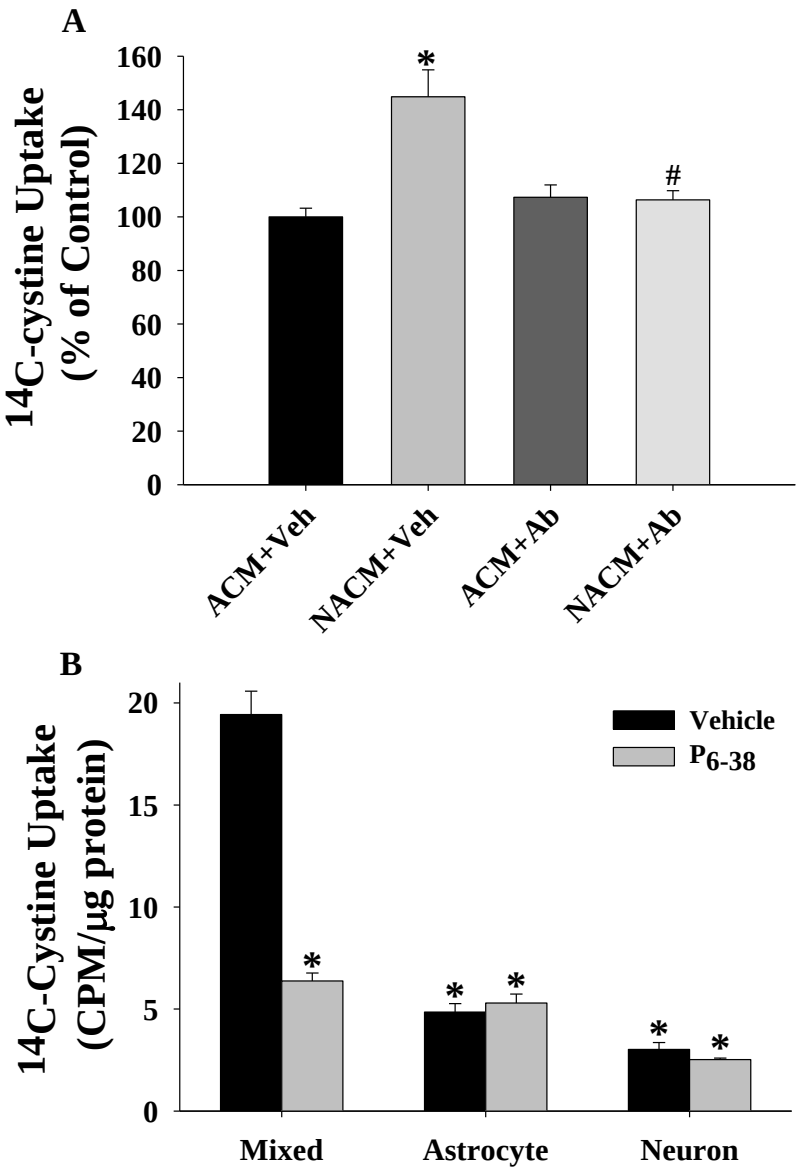

Figure 2.2. PACAP mediates neuronal regulation of Sxc activity. (A) Data depict mean ± SEM levels of ^{14}C-cystine in rat purified astrocyte cultures after 24 hours treatment with the media transferred from astrocytes (astrocyte-conditioned media, ACM) or astrocytes cocultured with neurons (neuron-astrocyte-conditioned media, NACM), with or without the presence of PACAP antiserum (Ab; N=6-12/condition). * indicates a significant difference relative to ACM treated cells that were not exposed to PACAP antiserum, T-test $p < 0.01$. # indicates a significant difference relative to NACM treated cells that were not exposed to PACAP antiserum, T-test $p < 0.01$. (B) Data depict the uptake of ^{14}C-cystine in rat cortical cultures in the presence or absence of the PACAP receptor inhibitor P6-38 (vehicle or 10 μM; N=6-12/condition). * indicates a significant difference relative to vehicle treated mixed cortical cultures, Bonferroni, $p < 0.001$.

As predicted, media from the astrocyte-neuron cocultures (NACM) increased Sxc activity (T-test, p <0.01), while removing free-PACAP from the NACM with PACAP-neutralizing antiserum significantly decreased Sxc activity (T-test, p <0.01). In addition, we examined the impact of inhibiting PACAP receptors with PACAP6-38 (P6-38) on ^{14}C-cystine uptake in rat mixed, purified astrocyte, and purified neuronal cortical cultures. A univariate ANOVA with cell composition and P6-38 treatment (0 or 10 μM) as between-subjects variables yielded a significant interaction [Figure 2.2B; $F(2,62)=73.849$, p < 0.001]. To deconstruct the interaction, we compared ^{14}C-cystine levels in vehicle-treated mixed cultures to every other condition. We found that vehicle-treated cultures containing neurons and astrocytes (i.e., mixed) displayed significantly higher levels of ^{14}C-cystine, and that this effect was blocked by inhibiting PACAP receptors with PACAP6-38 (P6-38) (Bonferroni, p < 0.001). Interestingly, P6-38 did not alter ^{14}C-cystine levels in purified astrocyte or in purified neuronal cultures. Together, these data suggest that PACAP mediates neuronal regulation of Sxc activity on astrocytes through activating PACAP receptors, and that the endogenous PACAP-induced upregulation of Sxc activity is only present in mixed cortical cultures (see Figure 2.2).

Experiment 3: PACAP up-regulates Sxc on astrocytes. To confirm that PACAP promotes Sxc activity in rat cortical astrocytes, we compared ^{14}C-cystine uptake in mixed cultures, purified astrocyte cultures, and purified neuronal cultures following PACAP application (24 hours) in the presence or absence of the Sxc inhibitor sulfasalazine (SSZ). A univariate ANOVA with culture cell composition and PACAP treatment as between subjects factors produced a significant interaction [Figure 2.3A; $F(4,62)=31.958$, p < 0.001]. To further analyze the data, we examined the impact of

PACAP on ^{14}C-cystine uptake in each type of culture. A significant simple main effect of PACAP treatment was obtained only in purified astrocyte cultures [$F(2,21)=75.904$, $p < 0.001$]. Subsequent *post hoc* analyses revealed that PACAP increased uptake at 10 and 100 nM (Bonferroni, $p < 0.001$).

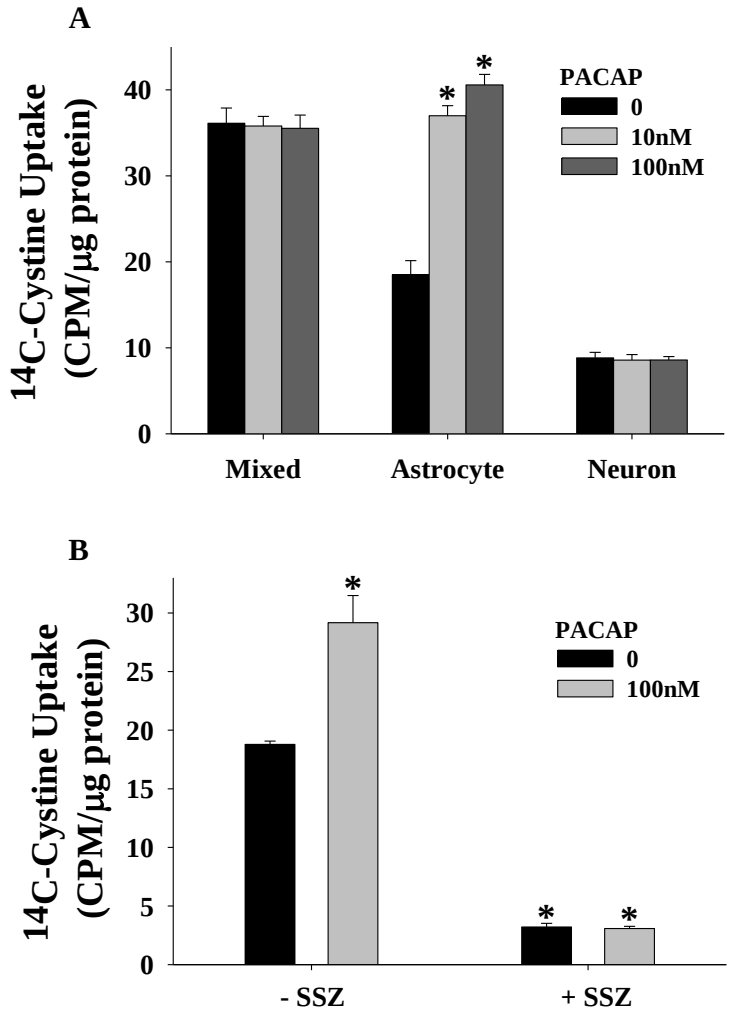

Figure 2.3. PACAP upregulates Sxc activity in cortical astrocytes only. (A) Data depict mean ± SEM levels of ^{14}C-cystine in rat cortical cell cultures after 24 hours PACAP treatment (0-100 nM; N=8/condition). * indicates a significant difference relative to vehicle-treated purified astrocyte cultures, Bonferroni, $p < 0.001$. (B) Data depict mean ± SEM levels of ^{14}C-cystine in rat purified astrocyte cultures after 24 hours PACAP (0 or 100 nM) assessed in the presence of an Sxc inhibitor sulfasalazine (SSZ, 0 or 300 µM; N=4/condition). * indicates a significant difference relative to PACAP vehicle treated cells that were not exposed to SSZ (-SSZ), Bonferroni $p < 0.001$.

Although Sxc is primarily associated with astrocytes, we verified that PACAP-induced increases in ^{14}C-cystine uptake reflect Sxc activity. To do this, we assessed the impact of PACAP on ^{14}C-cystine uptake by astrocytes in the presence and absence of the Sxc inhibitor SSZ. A univariate ANOVA with PACAP and SSZ treatments as between-subjects factors resulted in a significant interaction [Figure 2.3B; $F(1,12)=19.130$, $p < 0.01$]. *Post hoc* analyses indicated that PACAP increased ^{14}C-cystine uptake in astrocytes when tested in the absence (Bonferroni, $p < 0.001$), but not in the presence of SSZ.

Experiment 4: Cellular distribution of PACAP and PAC1R in rat cortical cultures. To further examine the possibility that PACAP is a neuronal factor capable of regulating astrocytes, we assessed the cellular expression patterns of PACAP and its primary receptor, PAC1R (Gottschall et al., 1990; Lam et al., 1990). A one-way ANOVA with culture cell composition as a between-subjects factor yielded a main effect on PACAP mRNA levels [Figure 2.4A; $F(2,17)=294.774$, $p < 0.001$]. *Post hoc* analyses revealed that mixed and purified neuronal cultures expressed the highest levels of PACAP mRNA (Bonferroni, $p < 0.001$). In fact, PACAP mRNA was not detectable in purified astrocyte cultures (Figure 2.4A). A one-way ANOVA comparing the cellular distribution of PAC1R mRNA yielded a main effect of cell type [Figure 2.4B; $F(2,17)=93.863$, $p < 0.001$]. *Post hoc* analyses revealed expression of PAC1R in every cell type, but with the highest levels evident in mixed cortical cultures (Bonferroni, $p < 0.001$). Collectively, these results demonstrate that in rat cortical cultures, PACAP is solely expressed in neurons and its primary receptor, PAC1R, is expressed by astrocytes.

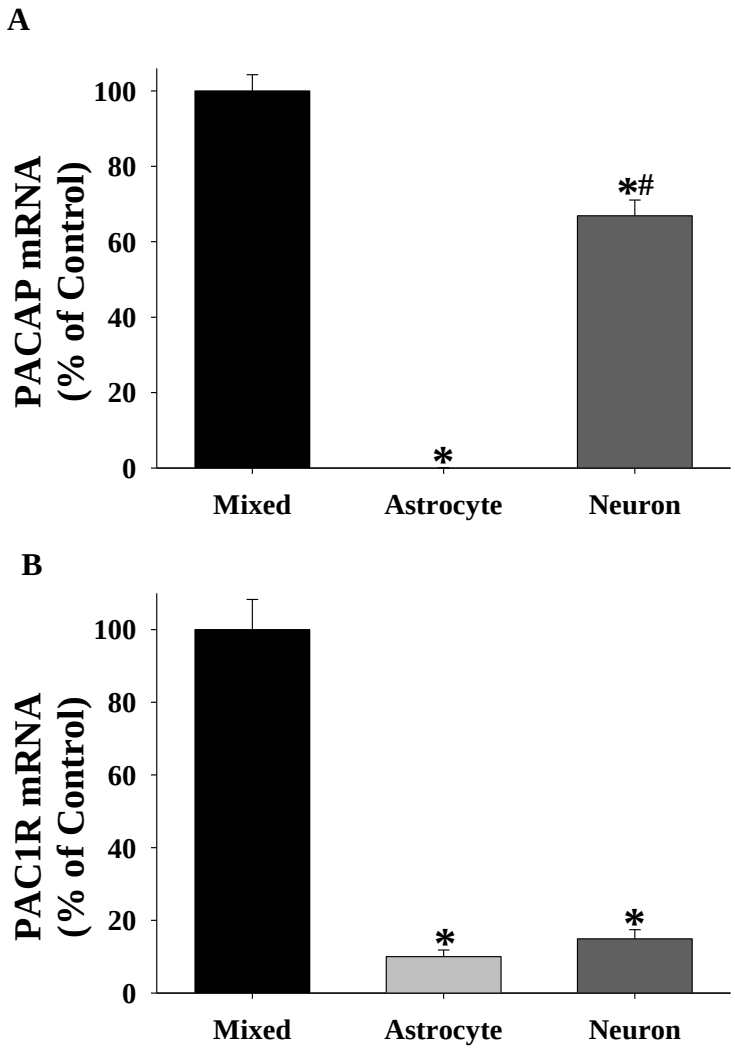

Figure 2.4. The cellular expression patterns of PACAP and its primary receptor, PAC1R, support the possibility that PACAP is a neuropeptide capable of regulating astrocyte Sxc activity. (A) Data depict mean ± SEM levels of PACAP mRNA in rat cortical cells cultures (N= 5-8/cell type). * indicates a significant difference relative to mixed cultures, Bonferroni, $p < 0.001$. # indicates a significant difference relative to purified astrocyte cultures, Bonferroni, $p < 0.001$. (B) Data depict mean ± SEM levels of PAC1R mRNA in rat purified astrocyte and neuronal cultures relative to that in cortical mixed cell cultures (N= 5-8/cell type). * indicates a significant difference relative to mixed cultures, Bonferroni, $p < 0.001$.

Experiment 5: Neurons and PACAP increase xCT mRNA. To better understand the observed upregulation of astrocyte Sxc activity by neurons or PACAP, we measured mRNA levels of xCT, the functional subunit of Sxc (Sato et al., 1999; Sato et al., 2000; Bridges et al., 2001). Mixed cultures of astrocytes and neurons [$t(6)=17.734$, *p*

< 0.001], as well as purified astrocyte cultures exposed to NCM [t(13)=13.431, $p < 0.001$] expressed significantly higher levels of xCT mRNA relative to respective controls (Figure 2.5). Similarly, PACAP application in purified astrocytes also robustly increased xCT mRNA [Figure 2.5; t(10)=16.554, $p < 0.001$]. Together, these results suggest that the upregulation of astrocyte Sxc by neurons or PACAP may involve increased Sxc expression.

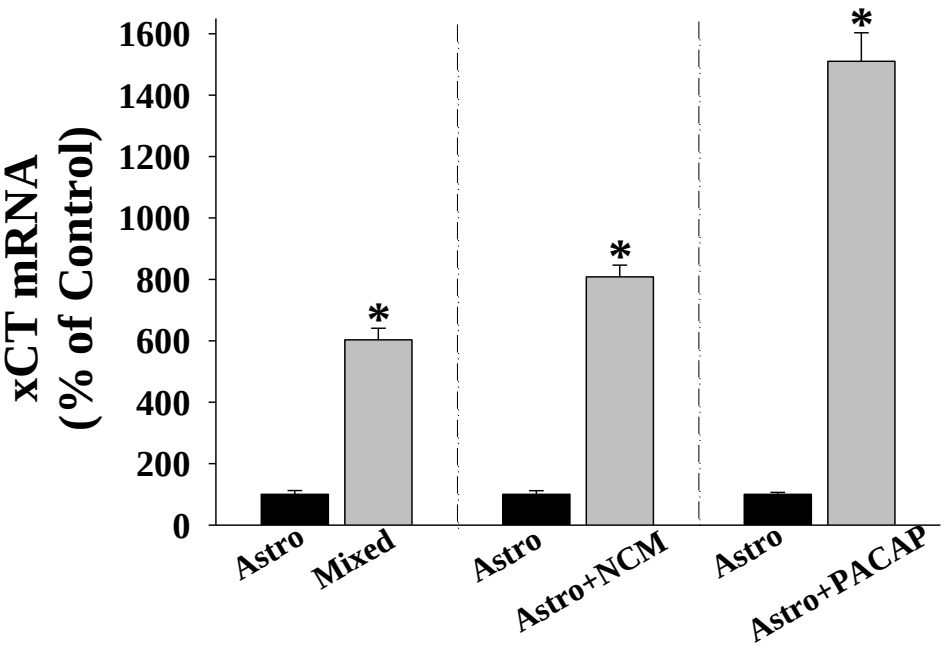

Figure 2.5. Exposure to neurons and PACAP increase xCT mRNA in cultured cortical astrocytes. Data depict mean ± SEM levels of xCT mRNA (normalized to respective controls) in rat cortical cells cultures (N=4-12/condition). Astro refers to purified astrocyte cultures; mixed cells refer to cultures comprised of neurons and astrocytes; NCM refers to neuronal conditioned media achieved by placing neurons grown on inserts into the culture wells containing astrocytes for 14 days; PACAP treatment was for 24 hours at 10 nM. * indicates a significant difference relative to the respective control, t-test, $p < 0.001$.

Discussion

Glutamate signaling is achieved by an elaborate network likely requiring coordinated activity between neurons and astrocytes. In support, both cells express glutamate receptors, transporters, and release mechanisms (Choi, 1988; Greenamyre et al., 1988; Coyle and Puttfarcken, 1993; Moghaddam and Adams, 1998; Araque et al., 1999; Tapia et al., 1999; Marino et al., 2001; Franks et al., 2003; Haydon and Carmignoto, 2006; Javitt et al., 2011; Santello et al., 2012; Araque et al., 2014; Perez-Alvarez et al., 2014). The purpose of these experiments was to determine whether, and how, neurons regulate components of the glutamate system expressed by astrocytes. To do this, we focused on system x_c- (Sxc) since this non-canonical release mechanism has been shown to be expressed primarily by astrocytes (Bannai and Kitamura, 1980, 1981; Sato et al., 1999; Pow, 2001; Zhang et al., 2014), significantly contributes to glutamate homeostasis (Baker et al., 2002; Xi et al., 2002; Melendez et al., 2005), regulates neuronal activity (Xi et al., 2002; Moran et al., 2005; Moussawi et al., 2009; Moussawi and Kalivas, 2010; Moussawi et al., 2011; Kupchik et al., 2012), and has been linked to multiple CNS diseases (Bridges et al., 2012a; Lewerenz et al., 2013; Deepmala et al., 2015). Our major finding is that Sxc activity is significantly increased when astrocytes are exposed to neurons or to neuronal factors, including pituitary adenylate cyclase-activating peptide (PACAP). These findings are significant, in part, because they illustrate a novel form of neuron to astrocyte communication. Hence, pathological changes involving components of the glutamate system expressed by astrocytes, such as Sxc, may stem from aberrant activity of neuronal circuits.

Neuronal Regulation of Sxc

In these studies, we found that the uptake of ^{14}C-cystine was higher in rat cortical cultures comprised of neurons and astrocytes relative to purified astrocyte cultures. One interpretation of these data is that neurons up-regulate Sxc activity in astrocytes. This conclusion, along with findings that neurons are critical in regulating the expression and activity of sodium-dependent glutamate transporters (Swanson et al., 1997; Figiel and Engele, 2000), highlights the need for neurons and astrocytes to display coordinated activity in order to achieve normal glutamate signaling. Alternative interpretations of our data include the possibility that neurons indirectly regulate Sxc activity. For example, neurons may influence the development of astrocytes, which may in turn influence Sxc.

In order to more directly test the hypothesis that a releasable neuronal factor coordinates Sxc activity on astrocytes, we evaluated the impact of neuronal conditioned media on ^{14}C-cystine uptake in cortical astrocytes. In this experiment, purified astrocyte cultures were conditioned to neurons through a non-contact co-culture system, in which neurons seeded on removable inserts were placed in astrocyte cultures for 14 days. Afterwards, the neuronal inserts were removed in order to measure ^{14}C-cystine uptake into astrocytes. Similar to our earlier finding, we found that cortical astrocytes that were exposed to neuronal conditioned media displayed significantly higher ^{14}C-cystine uptake. We did not observe any other difference in the astrocyte cultures exposed to neuronal conditioned media from those lacking neurons. For example, the protein counts (mean ± SEM: astrocytes, 267.1 ± 6.8 μg/ml; astrocytes exposed to NCM, 254.4 ± 6.0 μg/ml) or cell confluency did not differ. As a result, these findings support the conclusion that

neurons are capable of regulating components of the glutamate system expressed on astrocytes.

Regulation of Sxc by the neuropeptide PACAP

In an attempt to identify potential neuronal factors capable of regulating Sxc on astrocytes, we examined the possible involvement of PACAP since this peptide has been shown to be expressed by cortical neurons (Koves et al., 1991; Waschek et al., 1998; Figiel and Engele, 2000) and capable of regulating components of the glutamate system expressed by astrocytes including GLT1 and GLAST in rat cortical glial cultures and Sxc in mouse glial cultures (Figiel and Engele, 2000; Resch et al., 2014b). We sought to extend these studies by examining whether neuron-induced upregulation of ^{14}C-cystine uptake requires PACAP signaling. To do this, we took two separate approaches to disrupt signaling of PACAP in astrocytes. First, we sought to use neutralizing antiserum to remove PACAP from neuronal conditioned media (Figiel and Engele, 2000). In order to do this, however, we needed to modify the assay such that the compensatory changes involving PACAP release would not overwhelm PACAP antiserum. Therefore, instead of co-incubating astrocytes with neurons for 14 days, we applied neuronal conditioned media for 24 hours. As we predicted, media from neuron-astrocyte cocultures were capable of significantly increasing Sxc activity in astrocytes in a much shorter time period (e.g., 24 hours versus 14 days). This is important because it allowed us to examine the involvement of PACAP by neutralizing the potential PACAP content in the media with PACAP antiserum. Alternatively, blocking PACAP receptors with antagonists could also inhibit PACAP signaling. Application of PACAP6-38 (P6-38), a truncated version of PACAP that inhibits PACAP receptors including PAC1R and VPAC2R, and also CART

receptor (Robberecht et al., 1992; Gourlet et al., 1995; Laburthe et al., 2007; Hawke et al., 2009; Mounien et al., 2009; Lin et al., 2011), decreased ^{14}C-cystine uptake in mixed cortical cultures to the level observed in purified astrocyte cultures. Interestingly, P6-38 did not alter ^{14}C-cystine uptake in purified astrocytes or neurons. The results obtained in purified neuronal cultures likely reflect the relative lack of Sxc activity in these cells, although it should be noted that low amounts of ^{14}C-cystine are detected in these cells. The lack of a P6-38 effect in purified astrocytes indicates the lack of endogenous PACAP signaling in these cells.

In order to further evaluate the conclusion that PACAP is a neuropeptide capable of directly regulating astrocytes, we examined the cellular distribution of mRNA for PACAP and its primary receptor, PAC1R. PACAP mRNA expression in cortical cultures was restricted to neurons, thereby establishing PACAP as a neuropeptide in these cultures. In contrast, both cortical neurons and astrocytes express mRNA for PAC1R, which has been shown to be the most abundant PACAP receptor that is expressed in cortical astrocytes (Zhang et al., 2014). However, PACAP also stimulates VPAC1 and VPAC2 receptors, which are also expressed by astrocytes (Grimaldi and Cavallaro, 1999). Hence, there is a need to identify the receptor(s) that contributes to the regulation of Sxc by PACAP. Interestingly, we found significantly higher levels of PACAP and PAC1R in mixed cultures comprised of neurons and astrocytes, compared to either cell type cultured alone, which may suggest that astrocytes influence the neuronal PACAP expression. Yet, whether the upregulation of PAC1R reflects an increase in astrocytes or neurons is not clear. Notably, changes in mRNA do not always result in changes in protein expression and function. While future experiments are needed to further explore the potential for

astrocyte-neuron communication to be an important determinant of PACAP signaling, our extant results are consistent with the possibility that PACAP is a neuropeptide capable of regulating components of the glutamate system expressed by astrocytes.

Next, we directly tested the hypothesis that PACAP application increases Sxc activity in astrocytes. We found that PACAP application significantly increased ^{14}C-cystine uptake in rat cortical astrocytes, but not in mixed or purified neuronal cultures. While we anticipated PACAP-induced increases in ^{14}C-cystine uptake in rat cortical astrocytes and a lack of an increase in purified neuronal cultures since these cells generally display little to no Sxc activity, we expected a modest increase in the mixed cortical cultures, an outcome we previously observed in mouse cultures (Resch et al., 2014b). Aside from species, the difference between these studies is not clear, but likely reflects the existence of endogenous PACAP signaling. Collectively, these results are consistent with PACAP functioning as a neuronal factor controlling glutamate signaling involving astrocytes.

The nature of PACAP-induced regulation of Sxc in astrocytes is likely complex. For example, we found evidence that application of PACAP for 24 hours increased mRNA of xCT, the active subunit for Sxc. While increases in mRNA do not always result in augmented protein, it is important to note that PACAP also upregulated Sxc activity. Further, elevated xCT mRNA and increased Sxc activity were also evident in astrocytes cultured with neurons. Collectively, these data suggest that neurons influence the expression of Sxc in astrocytes. In addition, however, we found that relatively brief inhibition of PACAP signaling (i.e., 60 min) produced a significant reduction in Sxc

activity, which suggests the possibility of post-translational modification. Additional studies are needed to better understand neuron or PACAP-induced regulation of Sxc.

Altered glutamate signaling likely underlies, at least in part, many disorders of the brain. Hence, it is critical to better understand how coordinated activity across distinct cell types involved in the transmission of this amino acid, is achieved. For example, Sxc has been implicated in several CNS diseases (Baker et al., 2003; Chung et al., 2005; Madayag et al., 2007; Baker et al., 2008; Knackstedt et al., 2010; Bridges et al., 2012a; Lewerenz et al., 2013; Lutgen et al., 2013; Albano et al., 2015; Ching et al., 2015; Deepmala et al., 2015). Similarly, altered activity of GLT1 and other glutamate transporters expressed by astrocytes has also been implicated in pathological glutamate transmission (Soni et al., 2014; Jensen et al., 2015; Roberts-Wolfe and Kalivas, 2015). In each case, however, it is unclear how glutamate signaling involving astrocytes may be altered. Collectively, these studies indicate that aberrant neural activity may be a novel factor underlying pathological changes in glutamate stemming from altered regulation of astrocytes. Moreover, PACAP itself is especially interesting in this regard as it has been shown to regulate glutamate uptake and release by astrocytes.

CHAPTER III

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE-INDUCED REGULATION OF SYSTEM X_C- IN STRIATAL ASTROCYTES

Introduction

The previous chapter revealed that neurons regulate system x_c- (Sxc), a cystine-glutamate antiporter expressed primarily by astrocytes, through releasable factors. Moreover, this regulation appears to be mediated by the pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide expressed exclusively by neurons. This novel form of neuron-astrocyte interaction likely contributes to coordinated glutamate transmission in the brain, to which malfunctions have been indicated in numerous CNS disorders.

The regulation involved 24-hr PACAP application. Thus, it is unclear whether rapid, phasic changes in PACAP would regulate Sxc. This is important because phasic regulation is likely necessary for rapid control of synaptic transmission and behavior. At this point, the impact of PACAP is only observed in cortical astrocytes. Given our interest in understanding the regulation of NAc astrocytes, there is a need to determine whether these properties are restricted to the cortex. As such, in this chapter, we seek to expand our understanding of PACAP-induced regulation of Sxc in the cortex and striatum.

Three receptors, PAC1R, VPAC1R and VPAC2R, have been identified to mediate PACAP signaling (Vaudry et al., 2009). They are G-protein coupled receptors and are positively linked to Gαs (Harmar et al., 1998; Harmar, 2001). Therefore, activation of these receptors leads to stimulation of adenylyl cyclase (AC) and

intracellular accumulation of cAMP (Harmar, 2001). In addition, PAC1R is reportedly paired with more diverse transduction systems, including the activation of a Gαq-PLC cascade and the elevation of intracellular calcium (Arimura, 1998; Vaudry et al., 2000; Zhou et al., 2001; Dickson and Finlayson, 2009; Vaudry et al., 2009).

PKA and PKC are the two canonical kinases induced by Gαs and Gαq activations. Interestingly, sequence analyses of human xCT demonstrate two consensus PKA sites (Baker et al., 2002), and both PKA and PKC have been shown to influence Sxc activity (Gochenauer and Robinson, 2001; Baker et al., 2002; Tang and Kalivas, 2003). Taken together, PKA and PKC are potentially involved in PACAP-induced regulation of Sxc. Other cascades induced by PACAP include AC-cAMP-Epac, PI3K-AKT, MEK-ERK and CaMK pathways, some of which have been implicated in the regulation of Sxc and/or PACAP-induced regulation of astrocyte functions (Moroo et al., 1998; Vallejo and Vallejo, 2002; Hashimoto et al., 2003; Perez et al., 2005; Liu et al., 2012; Seo and Lee, 2016). Therefore, these pathways also potentially contribute to the actions of PACAP on Sxc.

In this chapter, we used purified astrocyte cultures derived from striatal tissues to expand our understanding of PACAP-induced regulation of Sxc. We found that PACAP upregulates Sxc in a dose- and time- dependent manner in both striatal and cortical astrocytes. Importantly, phasic PACAP significantly increases Sxc activity. In addition, we show the expression of all three PACAP receptor subtypes in both cortical and striatal astrocytes, with PAC1R and VPAC2R more abundantly expressed in astrocytes than in neurons. Lastly, our data suggest that the actions of PACAP on astrocyte Sxc are mediated by PKA, Epac and MEK/ERK.

Material and Methods

Animals and Materials: These experiments utilized cortical tissues obtained from Sprague Dawley rats (age was gestational day 15-16 or post-natal day 3-4; sex was undetermined; Envigo, Indianapolis, IN). Experimental procedures were approved by the Marquette University Institutional Animal Care and Use Committee. The primary materials included fetal bovine serum and horse serum (Atlanta Biologicals; Lawrenceville, GA), ^{14}C-cystine (PerkinElmer, Waltham, MA) and PACAP1-38 (PACAP; California Peptide Research, Napa, CA). All kinase activators and inhibitors were obtained from Sigma-Aldrich (Milwaukee, WI), including H89 (PKA inhibitor), Go6983 (PKC inhibitor), ESI09 (Epac inhibitor), KN93 (CaMKII inhibitor), LY294002 (PI3K inhibitor), U0126 (MEK inhibitor) and PD98059 (MEK inhibitor). All peptides were dissolved in saline, while all kinase inhibitors were reconstituted in DMSO.

Cell Culture Procedures: Purified astrocyte cultures were prepared from rat pups on post-natal day 3-4. In brief, cortical or striatal cells were disassociated and then suspended in Neurobasal A media (Thermo Fisher Scientific, Waltham, MA), supplemented with 10% fetal bovine serum, and 1% Glutamax (Thermo Fisher Scientific, Waltham, MA). Cells were initially grown in 25cm^2 or 75cm^2 cell culture flasks. Once confluent, cells were then subjected to prolonged, orbital shaking (250 rpm for 18 hours at 37^{o}C), which has been used to yield purified astrocyte cultures (McCarthy and de Vellis, 1980; Schildge et al., 2013). Purified astrocytes were then plated on 24-well plates, and refreshed with 70% new culture media every 2-3 days. Typically, it required 13-16 days *in vitro* (DIV13-16) for these cells to reach confluency, after which experiments were conducted.

Purified neuronal cultures were prepared from embryonic rat cortical (gestational day 15 to 16) tissues as previously described (Lobner, 2000), or striatal tissues (gestational day 17 to 18). In brief, dissociated cells were suspended in Eagles' Minimal Essential Medium (MEM, Earle's salts, glutamine-free), supplemented with glutamine (2mM), glucose (21mM), horse serum (5%), and fetal bovine serum (5%). Cells were seeded on 24-well plates. Forty eight hours later, cytosine arabinoside (at a final concentration of 10 μM) was added to the culture media to inhibit glial reproduction (Dugan et al., 1995). Neurons were then grown for an additional 11-13 days.

All cell-growing surfaces in culture flasks and 24-well plates were pre-coated with poly-D-lysine (10mg/L) and laminin (0.4mg/L). All cell cultures were maintained in humidified 5% CO_2 incubators at 37^{o}C.

^{14}C-cystine Uptake Assay: The assessment of system x_c- (Sxc) activity is often achieved by measuring intracellular uptake of radiolabeled cystine, since this is primarily dependent on Sxc (Liu et al., 2009; Liu et al., 2014a; Resch et al., 2014b; Albano et al., 2015). As demonstrated previously, over 85% of ^{14}C-cystine uptake in our cells was blocked by the Sxc inhibitor sulfasalazine (SSZ; Chapter II, Figure 2.3B). Radiolabeled cystine uptake assays were performed as described previously with minor modifications (Liu et al., 2009). In brief, experiments were conducted in a bead bath at 37^{o}C. Cells were washed 3 times with warm HEPES buffered saline solution, after which ^{14}C-cystine was added to the media for 20 min at a final concentration of 0.3μM. This concentration was used since it is similar to extracellular cystine concentrations in the brain (Baker et al., 2003). DL-threo-β-hydroxyaspartic acid (TBOA; 10 μM) was added to the culture media to prevent ^{14}C-cystine uptake by sodium-dependent glutamate transporters. After

incubating cells with ^{14}C-cystine, cells were washed 3 times with ice-cold HEPES buffered saline solution, and then solubilized with 1M NaOH solution. One aliquot of cell lysate was used for protein determination using the bicinchoninic acid (BCA) assay, and another aliquot was used for scintillation counting to measure the level of ^{14}C-cystine uptake. ^{14}C-cystine content was normalized to protein concentration. Data are presented as CPM/μg of protein or normalized to respective controls.

RNA extraction and cDNA construction: Total RNA was isolated from cell cultures with Trizol Reagent (Invitrogen; Carlsbad, CA) following the manufacture's protocol. DNase treatment was applied to all RNA samples to remove potential genomic DNA contamination with a DNA-free kit (Life Technologies, Carlsbad, CA). Assessment of RNA purity and quantity was performed on a NanoVue Plus Spectrophotometer (GE life sciences; Pittsburg, PA). cDNA was constructed from total RNA using the Reverse Transcription System (Promega, Madison, WI) with oligo(dT) primers following the manufacturer's protocol.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis: PCR were performed with GoTaq Green Master Mix (Promega, Madison, WI) on a thermocycler with the following parameters: initial denaturation (3min at 95 ℃), amplification (35 cycles of the following: 95 ℃ for 30s, 60 ℃ for 45s, 72 ℃ for 45s), and extension (72 ℃ for 10min). PCR products were then examined with electrophoresis on an agarose gel (1.8%) along with a 100bp DNA Ladder (Promega, Madison, WI) to locate target PCR products.

Real-time quantitative PCR: Quantitative PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems; Carlsbad, CA) and PerfeCTa SYBR Green

FastMix with ROX (Quanta Biosciences; Gaithersberg, MD). Relative quantification of target gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). Primer sequences were as follows. xCT (catalytic subunit of Sxc) forward- 5' AGG GCA TAC TCC AGA ACA CG 3'; xCT reverse- 5' ATG CTC GTA CCC AAT TCA GC 3'; PAC1R forward- 5' TGC CTG TGG CTA TTG CTA TG 3'; PAC1R reverse- 5' TTT AGT CCC ATC AGG TCG TTG 3'; VPAC1R forward-5' TGA GAT ACA GCG TCA GCA GTG 3'; VPAC1R reverse- 5' GCA AAC AGC TGA AAG ATG AGG 3'; VPAC2R forward-5' TGG CAA TGA CCA GTC ACA GT 3'; VPAC2R reverse- 5' CCT GGA AGG AAC CAA CAC AT 3'; GAPDH forward- 5' CTC CCA TTC TTC CAC CTT TGA 3'; GAPDH reverse- 5' ATG TAG GCC ATG AGG TCC AC 3'. Primers were designed to be intron-spanning using the online primer design tool Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). A single amplified product was confirmed by melt curve analysis. Amplification efficiency of all genes was determined to be approximately 95%.

Statistics: Statistical analyses were performed using SPSS Statistics (Version 24, IBM; Armonk, New York). Analysis of variance (ANOVA) was used when comparing data sets that included more than two groups. Tukey HSD tests were used for subsequent *post hoc* analyses of significant effects involving more than two groups. Student's t-tests were used when comparing results from only two groups. In all instances, statistical significance was designated as $p < 0.05$.

Results

Experiment 1: PAC1R and VPAC2R are enriched in cultured astrocytes obtained from striatal tissues. Since Sxc in the nucleus accumbens (NAc, ventral striatum) is critical for motivated behavior (Baker et al., 2003; Kau et al., 2008; Berglind et al., 2009; Kalivas, 2009; Knackstedt et al., 2009; Kupchik et al., 2012), we were interested in examining whether PACAP would regulate Sxc in striatal astrocytes. To do this, we first sought to determine which PACAP receptors these cells express. To date, three PACAP receptors, including PAC1R, VPAC1, and VPAC2R, have been identified. Therefore, we investigated the expression of these receptors in cortical and striatal astrocytes; cortical astrocytes were used as positive controls as they have been shown to express all these receptors (Zhang et al., 2014). Using PCR, we detected mRNAs of all three receptors in both cortical and striatal astrocytes. With a more stringent quantitative method, RT-qPCR, we investigated the relative levels of PAC1R and VPAC2R in striatal cell cultures (Figure 3.1B, C). These two PACAP receptors are selected because they have been shown to be significantly more abundant than VPAC1R in astrocytes, with FPKM (fragments per kilobase of transcript per million mapped reads) values to be > 150 (PAC1R), < 0.2 (VPAC1R) and ≈ 2 (VPAC2R) (Zhang et al., 2014). Moreover, both PAC1R and VPAC2R appear to be more enriched in astrocytes than in neurons, with ratios at 10:1 (PAC1R) and 5:1 (VPAC2R), while VPAC1R is more enriched in neurons, with a 5:1 ratio compared to astrocytes (Zhang et al., 2014). Consistently, we found that striatal astrocytes express significantly higher levels of mRNA for both PAC1R [Figure 3.1B; $t(6)=5.618$, $p < 0.01$] and VPAC2R [Figure 3.1C; $t(6)=11.082$, $p < 0.001$], compared to neurons of the same origin.

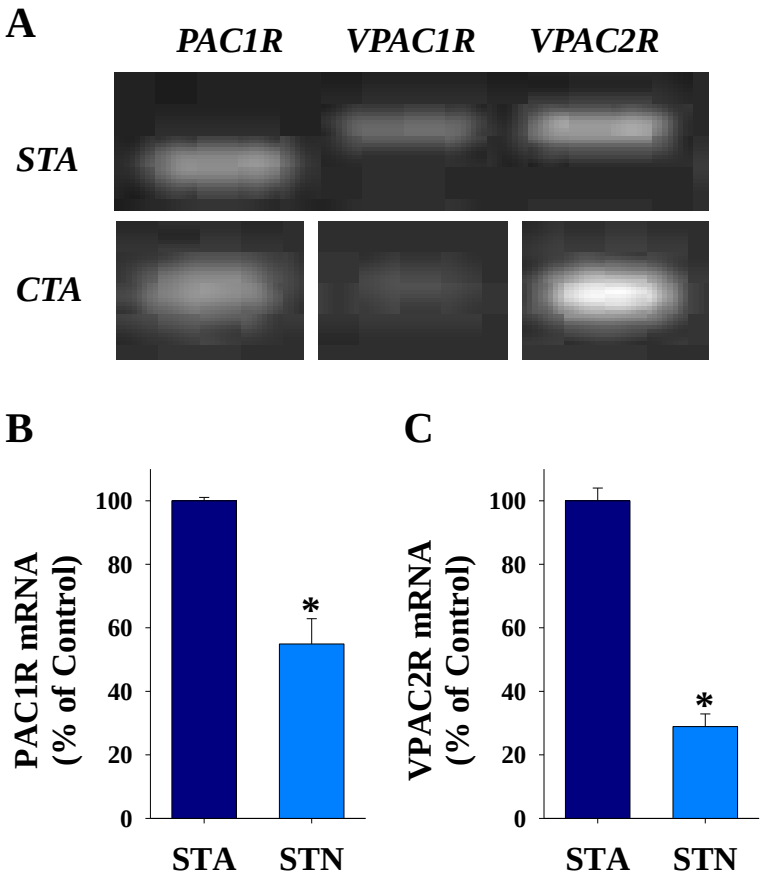

Figure 3.1. PAC1R and VPAC2R are enriched in cultured astrocytes obtained from striatal tissues. (A) Pictures illustrate the PCR products on agarose gels (1.8%) after electrophoresis. cDNA was synthesized through reverse transcription with mRNA extracted from cortical or striatal astrocyte cultures: CTA, cortical astrocytes; STA, striatal astrocytes. PCR was conducted with cDNA templates and primers specific to rat PAC1R, VPAC1R and VPAC2R. Presence of bands at expected sizes indicates the expression of the target genes. (B, C) Data depict mean ± SEM levels of PAC1R (B) or VPAC2R (C) mRNA in rat striatal cell cultures (N= 4/cell type). STN, striatal neurons. * indicates a significant difference relative to STA cultures, T-test, $p < 0.01$.

Experiment 2: Twenty-four hour PACAP incubation increases Sxc activity in striatal astrocytes. Having observed the expression of PACAP receptors in striatal astrocytes, we next examined the impact of PACAP on Sxc. In Chapter II, we demonstrated that 24-hr PACAP treatment significantly increased Sxc activity in cortical

astrocytes. Therefore, we investigated whether the same treatment would also impact striatal astrocytes (Figure 3.2). One-way ANOVAs revealed a main effect of PACAP concentration on the uptake levels in either cortical [Figure 3.2A; $F(4,43)=82.563$, $p < 0.001$] or striatal [Figure 3.2B; $F(4,47)=18.04$, $p < 0.001$] astrocytes, suggesting that PACAP dose-dependently increases astrocyte Sxc activity, independent of cell origins.

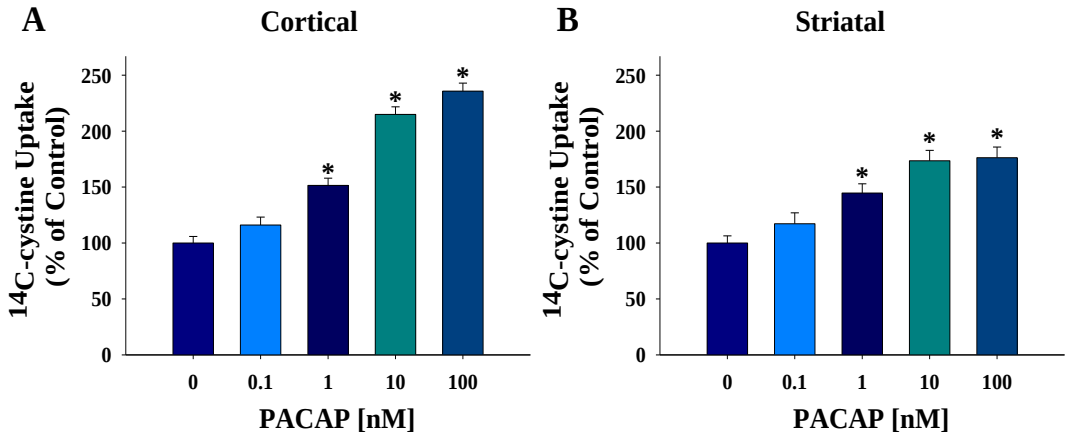

Figure 3.2. Twenty-four hour PACAP incubation increases Sxc activity in striatal astrocytes. Data depict mean ± SEM levels of ^{14}C-cystine in purified rat cortical (A) or striatal (B) astrocyte cultures (14-15 days *in vitro*) after incubation with PACAP for 24 hours (0-100 nM; N=8-16/condition). * indicates a significant difference relative to vehicle-treated group, Tukey HSD, $p < 0.01$.

Experiment 3: Inhibition of PKA or Epac attenuates the upregulation of Sxc activity by 24-hr PACAP treatment in striatal astrocytes. Each of the PACAP receptors is coupled to Gαs and/or Gαq. As such, PKA and PKC have been primarily implicated in the intracellular actions of PACAP (Miyata et al., 1989; Lazarovici et al., 1998; Figiel and Engele, 2000; Zhou et al., 2001). However, PACAP has also been shown to signal through AC-cAMP-Epac, PI3K-AKT, MEK-ERK or CaMK pathways (Moroo et al., 1998; Vallejo and Vallejo, 2002; Hashimoto et al., 2003; Perez et al., 2005; Blechman and Levkowitz, 2013). To determine the involvement of these potential signaling cascades in PACAP-induced regulation of Sxc, we examined the impact of

inhibiting respective kinases. Specifically, we pre-incubated striatal astrocytes for one hour with the following kinase inhibitors: H89 (PKA inhibitor), Go6983 (PKC inhibitor), ESI09 (Epac inhibitor), KN93 (CaMKII inhibitor), LY294002 (PI3K inhibitor) or U0126 (MEK inhibitor). Vehicle or PACAP (10nM) was then added to the cultures for an additional 24 hours (Figure 3.3). A one-way ANOVA comparing the ^{14}C-cystine uptake

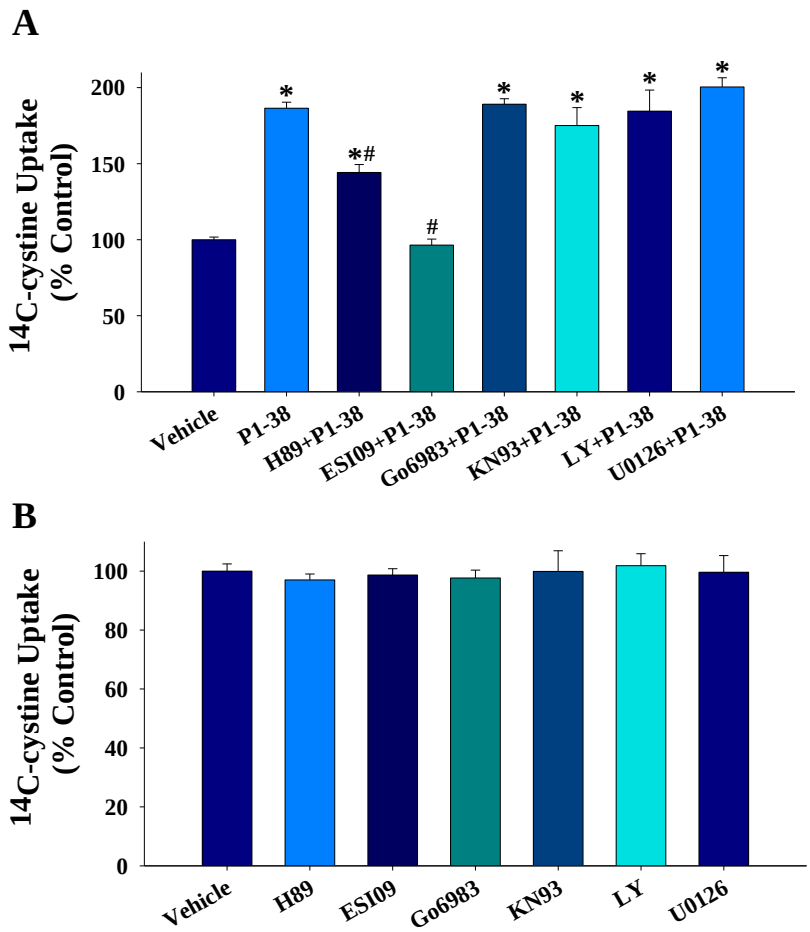

Figure 3.3. Inhibition of PKA or Epac attenuates the upregulation of astrocyte Sxc by 24-hr PACAP treatment. Data depict mean ± SEM levels of ^{14}C-cystine in purified rat striatal astrocyte cultures after 24-hr treatment with vehicle or PACAP 10nM (designated as P1-38), with or without the presence of H89 (10 µM, PKA inhibitor), ESI09 (5 µM, Epac inhibitor), Go6983 (1 µM, PKC inhibitor), KN93 (5 µM, CaMKII inhibitor), LY294002 (designated as LY, 45 µM, PI3K inhibitor), U0126 (25 µM, MEK inhibitor) (N=4-14/condition). Note that the kinase inhibitors were added one hour prior to PACAP application (A). * indicates a significant difference relative to vehicle-treated purified astrocyte cultures, Tukey HSD, $p < 0.001$; # indicates a significant difference relative to PACAP treated purified astrocyte cultures, Tukey HSD, $p < 0.001$.

levels yielded a main effect of treatment [Figure 3.3A; $F(7,36)=61.130$, $p < 0.001$]. *Post hoc* analyses indicate that presence of H89 and ESI09 significantly reduced PACAP-induced Sxc activity (Tukey HSD, $p < 0.001$), suggesting that the effect of 24-hr PACAP is mediated by Gαs-AC-cAMP-PKA/Epac. Note, we observed that applying the kinase inhibitors alone to astrocyte cultures did not alter cystine uptake compared to the vehicle group (Figure 3.3B).

Experiment 4: Phasic PACAP upregulates Sxc activity in a time- and dose-dependent manner in striatal astrocytes. In these studies, we were interested in investigating whether short-term application of PACAP would regulate Sxc, given that phasic regulation is likely involved in the rapid control of synaptic transmission and behavior. To examine this, we applied 10 nM PACAP to cortical or striatal astrocytes cultures (DIV 14-15) for 6 or 20 min, prior to ^{14}C-cystine uptake assays (Figure 3.4A).

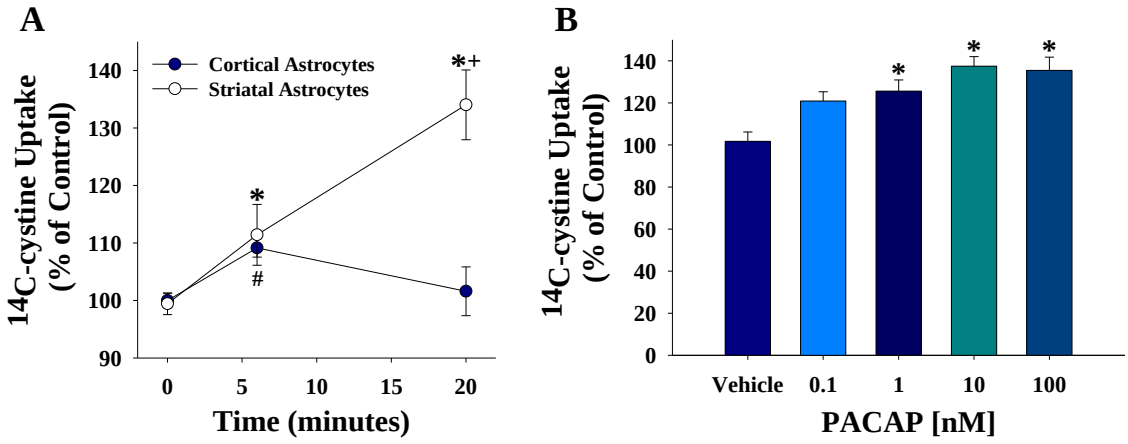

Figure 3.4. Time- and dose-dependent regulation of Sxc activity by short-term application of PACAP in striatal astrocytes. (A) Data depict mean ± SEM levels of ^{14}C-cystine in rat cortical or striatal astrocyte cultures after 6 or 20 min treatment with vehicle (designated as 0 min group) or PACAP (10 nM; N=4-38/condition). , * indicates a significant difference relative to the vehicle group of striatal cultures, Tukey HSD, $p < 0.05$; # indicates a significant difference relative to the vehicle-treated cortical cultures, Tukey HSD, $p < 0.01$; + indicates a significant difference relative to 20 min PACAP-treated cortical cultures, T-test, $p < 0.01$. (B) Data depict mean ± SEM levels of ^{14}C-cystine in striatal astrocyte cultures after incubation with PACAP for 20 min (0-100 nM; N=8/condition). * indicates a significant difference relative to vehicle-treated group, Tukey HSD, $p < 0.05$.

This concentration was selected because of its effectiveness in inducing an Sxc increase (see Figure 3.2). A univariate ANOVA with cell origin and incubation time as between-subjects factors produced a significant interaction between these variables. Further analyses revealed that PACAP time-dependently increased Sxc activity in both cortical [One-way ANOVA; $F(2,55)=8.862$, $p < 0.01$] and striatal astrocytes [One-way ANOVA; $F(2,33)=18.405$, $p < 0.001$]. Notably, twenty minute incubation yielded a higher level of Sxc activity in striatal astrocytes compared to cortical cultures [$t(10)=4.397$, $p < 0.01$].

Next, we investigated the dose-dependent effect of phasic PACAP. We chose 20 min as incubation time as it yields a more robust Sxc activity increase than 6 min, as well as a higher level than the cortical group, and also fits the behavior time frame (two hours) that we often use to measure drug seeking behaviors. One-way ANOVAs revealed an effect of PACAP concentration on cystine uptake [Figure 3.4B; $F(4,35)=8.015$, $p < 0.001$], indicating that 20-min PACAP incubation dose-dependently increases Sxc activity in striatal astrocytes. These results indicate that phasic PACAP augments Sxc activity in a time- and dose-dependent manner in striatal astrocytes, suggesting the occurrence of this regulation in the NAc.

Experiment 5: The impact of PACAP on Sxc is reversed by inhibiting PKA, Epac and MEK pathways. Next, we sought to explore the involvement of potential signaling cascades, as introduced above, in the rapid regulation of Sxc. To do this, we first applied PACAP to striatal astrocytes for 72 hr followed by the addition of the kinase inhibitors during the last hour of the PACAP treatment period. A one-way ANOVA comparing the ^{14}C-cystine uptake levels yielded a main effect of treatment [Figure 3.5; $F(7,36)=74.607$, $p < 0.001$]. *Post hoc* analyses revealed that H89 and ESI09 significantly

reduced the upregulation of Sxc activity induced by PACAP (Tukey HSD, $p < 0.001$). Interestingly, blocking the MEK/ERK pathway with PD98059 also attenuated PACAP-induced upregulation of Sxc activity, suggesting a differential role of this pathway in acute PACAP-induced regulation of Sxc.

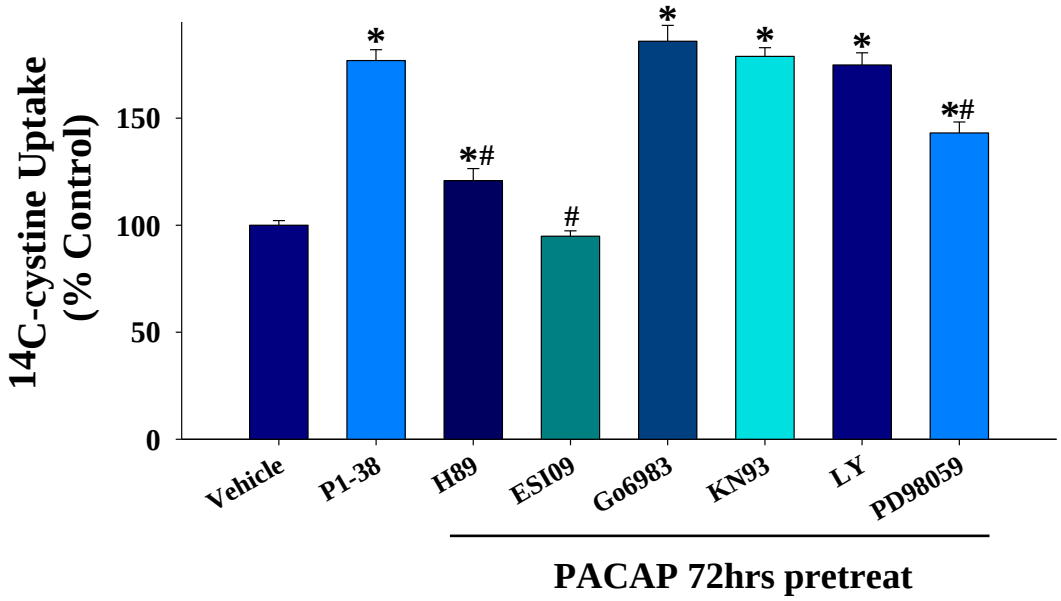

Figure 3.5. PKA, Epac and MEK pathways are involved in the rapid regulation of Sxc activity by PACAP. Data depict mean ± SEM levels of ^{14}C-cystine in purified rat striatal astrocyte cultures. Prior to ^{14}C-cystine uptake assay, cells were incubated with vehicle or 10nM PACAP (designated as P1-38) for 72 hours. Kinase inhibitors, H89 (10 µM), ESI09 (5 µM), Go6983 (1 µM), KN93 (5 µM), LY294002 (designated as LY, 45 µM), or PD98059 (50 µM, MEK inhibitor) was applied and present only for the last hour during PACAP incubation (N=4-12/condition). * indicates a significant difference relative to vehicle-treated purified astrocyte cultures, Tukey HSD, $p < 0.05$. # indicates a significant difference relative to PACAP treated purified astrocyte cultures, Tukey HSD, $p < 0.001$.

Discussion

The primary goal for these studies was to determine whether Sxc is modulated by PACAP in regions other than the cortex and to better understand the nature of this novel form of neuron-astrocyte communication. The major findings in these data are that both

prolonged and phasic PACAP upregulate Sxc in astrocytes obtained from either rat cortex or striatum. We also found that two major subtypes of PACAP receptors, PAC1R and VPAC2R, are enriched in astrocytes compared to neurons. Consistent with the evidence that these receptors are commonly coupled to Gαs (Harmar, 2001; Vaudry et al., 2009), our data suggest that two major Gαs-induced kinases, PKA and Epac, mediate the actions of PACAP.

To date, PACAP-induced regulation of Sxc has only been detected in cortical cells (Resch et al., 2014b; Kong et al., 2016). However, we are interested in understanding the molecular regulation of Sxc in astrocytes obtained from the striatum. This brain region is of particular interest because Sxc function in the nucleus accumbens (NAc, ventral striatum) is critical for the proper expression of motivated behaviors, whereas malfunction of this transporter contributes significantly to the development or maintenance of substance abuse (Baker et al., 2003; Kau et al., 2008; Berglind et al., 2009; Kalivas, 2009; Knackstedt et al., 2009; Kupchik et al., 2012). To examine this, we first compared the impact of 24-hour PACAP treatment on cystine uptake, in part so that we could compare our results with prior observations from cortical astrocytes. We found that PACAP yielded similar effects on striatal astrocytes to those from the cortex, suggesting that PACAP-induced regulation of Sxc is also present in the striatum. This raises the prospect that PACAP-induced regulation of astrocyte Sxc may be a common feature in the brain. This is important, since Sxc is an important component of glutamate signaling, and has been found to regulate synaptic transmission (Manzoni et al., 1997; Baker et al., 2002; Moran et al., 2005; Augustin et al., 2007; Moussawi and Kalivas, 2010; De Bundel et al., 2011; Massie et al., 2011; Williams and Featherstone, 2014). Moreover,

Sxc is expressed in various regions of the CNS, including the amygdala, hippocampus and spinal cord (Roberto and Brunelli, 2000; De Bundel et al., 2011; Albano et al., 2013; Aal-Aaboda et al., 2015), where PACAP signaling has been implicated (Moller et al., 1993; Zhang et al., 1995; Roberto and Brunelli, 2000; Cho et al., 2012). As such, this evidence creates a need to better understand the contribution of this novel form of neuron-astrocyte communication to the excitatory signaling in these brain regions and related CNS functions, including memory, emotion and motor generation.

Next, we determined whether PACAP could produce rapid regulation of Sxc in a manner that would be consistent with the regulation by phasic changes in PACAP. This is important because phasic regulation is likely necessary for rapid control of synaptic transmission and behavior. We found that PACAP application for as short as 6 min induced a significant upregulation of Sxc activity in striatal astrocytes. Given that neuropeptides (e.g. PACAP) are often released in a phasic manner (Nusbaum et al., 2001; van den Pol, 2012), this finding implicates that activity-dependent phasic release of PACAP in the striatum is capable of increasing Sxc, through which to regulate synaptic transmission and motivated behavior.

In these studies, we found that cortical and striatal astrocytes express all three receptor subtypes that mediate PACAP signaling, PAC1R, VPAC1R and VPAC2R (Harmar, 2001; Vaudry et al., 2009), consistent with earlier reports (Ashur-Fabian et al., 1997; Magistretti et al., 1998; Grimaldi and Cavallaro, 1999; Joo et al., 2004; Masmoudi-Kouki et al., 2007; Nishimoto et al., 2007; Nishimoto et al., 2011; Zhang et al., 2014). These receptors bind to PACAP with similarly high affinities and are reportedly expressed at differently levels in the CNS. For example, PAC1R, the major binding site

for PACAP (Dejda et al., 2011), appears to be the most abundant subtype in the cortex, while the VPAC1R appears to be the least expressed subtype (Zhang et al., 2014). For example, the FPKM (fragments per kilobase of transcript per million mapped reads) values of these three receptors in astrocytes are > 150 (PAC1R), < 0.2 (VPAC1R) and ≈ 2 (VPAC2R) (Zhang et al., 2014). Moreover, both PAC1R and VPAC2R appear to be more enriched in astrocytes than in neurons, with ratios at 10:1 (PAC1R) and 5:1 (VPAC2R), while VPAC1R is more enriched in neurons, with a 5:1 ratio compared to astrocytes (Zhang et al., 2014). Interestingly, we also show that both PAC1R and VPAC2R, the more abundant subtypes, are more enriched in striatal astrocytes compared to neurons. Together with our previous observation that PACAP6-38 (antagonist for both PAC1R and VPAC2R) in mixed rat cortical cultures significantly decreased Sxc activity (Chapter II, Figure 2.2), this evidence suggests that PAC1R and VPAC2R may be major receptors mediating the PACAP effects on astrocyte Sxc.

Activation of PAC1R and VPAC2R induces Gαs and Gαq, and subsequently a number of interconnected signaling cascades (Dickson and Finlayson, 2009; Vaudry et al., 2009; Blechman and Levkowitz, 2013). To tease out those that were most likely involved in the current studies, we chose the major pathways that were reported to mediate PACAP-induced regulation of astrocytes, including those that are involved in regulating Sxc (Moroo et al., 1998; Vallejo and Vallejo, 2002; Hashimoto et al., 2003; Perez et al., 2005; Seo and Lee, 2016). With this strategy, we first examined the involvement of PKA, PKC, Epac, CaMKII, PI3K/Akt and MEK/ERK in the action of 24-hr PACAP, as this time point yields the most robust effect on Sxc activity. Our data show that inhibiting cAMP-responding kinases, either PKA or Epac, significantly attenuated the effect of

PACAP in striatal astrocytes. In contrast, inhibition of two major kinases induced by Gαq, PKC and CaMKII, had no impact on PACAP effect (Figure 3.3). Thus, our results suggest that the Gαs- but not the Gαq- induced cascades mediate the actions of PACAP on astrocyte Sxc. This is consistent with a number of studies showing that Gαs-AC-cAMP-PKA/Epac pathway mediates PACAP-induced regulation of astrocyte functions (Figiel and Engele, 2000; Masmoudi et al., 2003; Seo and Lee, 2016).

In these studies, we also examined the impact of acutely inhibiting the potentially involved kinases (Figure 3.5). To our surprise, we found that inhibiting either PKA or Epac for one hour significantly attenuated the effect of PACAP (by approximately 75% and 100% respectively). In this experiment, striatal astrocytes were incubated with PACAP for 72 hours, for the purpose of maximizing the activity of Sxc. This treatment likely induces increased xCT and subsequently Sxc expression. In support, we showed that 24-hr PACAP incubation robustly upregulates xCT transcripts (Kong et al., 2016). However, the increased Sxc activity induced by 72-hr PACAP is probably not a mere reflection of increased Sxc expression. This is because that inhibiting the involved kinase Epac for only one hour, which unlikely induces a complete turnover of the increased Sxc expression, completely abolished the effect of PACAP. A feasible explanation is that, for the newly synthesized Sxc to gain function, they must undergo certain rapid molecular processes (e.g. post-translational modifications), which are also regulated by PACAP, and that the prolonged PACAP (i.e. 72 hr)-induced upregulation of Sxc activity results from both increased Sxc expression and augmented molecular processes that render Sxc function. Based on this presumption, our data suggest that cAMP-induced cascades (i.e. PKA and Epac) are also important for mediating the rapid effect of PACAP on Sxc.

Interestingly, PI3K/Akt and MEK/ERK, which were shown to mediate Sxc expression (Liu et al., 2012), did not demonstrate their involvement in the actions of 24-hr PACAP (Figure 3.3); yet inhibiting MEK/ERK for one hour after 72-hr PACAP incubation significantly decreased Sxc activity. These results suggest that the MEK/ERK pathway is only involved in mediating the rapid impact of PACAP on Sxc.

Although H89 is commonly used as a PKA inhibitor, it has been shown to inhibit other kinases, including MSK (Lochner and Moolman, 2006). It is intriguing to note that MSK is a downstream kinase of both PKA and Epac (Delghandi et al., 2005; Shi et al., 2012), and sequence analyses reveal that xCT harbors phosphorylation sites for MSK, including amino acid serine 26 (S26). This is important, because it has been recently demonstrated that mutagenesis of S26 on xCT and consequently altering phosphorylation state of the protein lead to changes of Sxc (McClatchy et al., 2016). Such evidence implicates MSK as a potential kinase that could mediate the effect of PKA and Epac on Sxc phosphorylation. Alternatively, PKA itself may also influence Sxc activity through direct phosphorylation of xCT, given that this catalytic subunit also harbors phosphorylation sites for PKA (Baker et al., 2002).

To summarize, we expanded our previous findings by showing that PACAP regulates Sxc in striatal astrocytes, implicating the occurrence of this mechanism in the NAc. Moreover, we observed a rapid upregulation of Sxc activity induced by phasic PACAP. Lastly, we found that two cAMP-regulated signaling pathways, PKA and Epac, may underlie PACAP-induced increases in Sxc activity. These findings indicate that PACAP potentially regulates glutamate transmission in the NAc via its actions on Sxc, and consequently influences motivated behaviors.

CHAPTER IV

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE PROMOTES GLUTAMATE RELEASE FROM ASTROCYTE SYSTEM X_C^- TO GATE SYNAPTIC OUTPUT FROM THE NUCLEUS ACCUMBENS

Introduction

Drug addiction is a complex, multi-faceted disorder characterized by compulsive drug seeking even when such behavior results in profound negative consequences to the individual. The chronic aspect of the disease involves episodes of heightened relapse vulnerability even following long periods of abstinence (Jaffe et al., 1989; Withers et al., 1995). Studies suggest that relapse originates from the persistent neuroadaptations at the molecular and cellular levels in the related brain circuit (Kalivas and Volkow, 2005; Nestler, 2005; Hyman et al., 2006; Koob and Volkow, 2010). Among these neuroadaptations, drug-induced changes in synaptic transmission due to aberrant glutamate signaling in the nucleus accumbens (NAc) and related circuitry have been linked to behavior in virtually every preclinical model of drug seeking and have been found to correlate with the severity of drug craving in human substance abusers (Pierce et al., 1996; Breiter et al., 1997; Volkow et al., 1999; Park et al., 2002; Baker et al., 2003; McFarland et al., 2003; McFarland et al., 2004; Schmidt et al., 2005; Madayag et al., 2007; Kau et al., 2008; Ary et al., 2013). However, a challenge in revealing the molecular or cellular basis of drug-induced plasticity is that excitatory neurotransmission is achieved by an elaborate network of glutamate-related proteins expressed across multiple cell types, including neurons and astrocytes (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997; Danbolt, 2001; Schoepp, 2001; Baker et al., 2002; Baker et al.,

2003; Pirttimaki et al., 2011; Bridges et al., 2012a), and that this signaling occurs in and outside of the synaptic cleft. Hence, it has been difficult to develop a comprehensive model that mechanistically integrates disparate forms of plasticity spanning multiple cell types. Of particular importance, it would be very useful to decode the molecular basis for integrating glutamate-related processes across cell types.

Here, we pursue the idea that the molecular integration of the cellular network underlying glutamate signaling in the NAc is achieved by the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP). Our interest in PACAP stems from emerging results establishing this peptide as a powerful regulator of excitatory signaling capable of regulating neuronal and astrocytic glutamate-related mechanisms. In support, published reports demonstrate that PACAP signaling in hippocampal and SCN (suprachiasmatic nucleus) neurons regulates AMPAR-mediated EPSCs (Kopp et al., 2001; Costa et al., 2009), an effect that may be dependent on the activation of high-affinity extrasynaptic receptors (Toda and Huganir, 2015). Moreover, we and others have discovered that PACAP can upregulate the activity of Sxc (Resch et al., 2014b; Kong et al., 2016), even when briefly applied (Chapter III). Collectively, these studies provide support for the proposed hypothesis that PACAP integrates astrocytes and neurons in a manner that regulates synaptic transmission and behavior.

To test our hypothesis, we first had to determine whether *in vivo* PACAP signaling exists in the adult NAc. It is important to determine whether PACAP and PACAP receptors are expressed in the adult NAc since many glutamate related proteins show drastic development-induced changes (Sun et al., 2013). To do this, we determined whether PACAP mRNA is present in excitatory projections to the NAc and if astrocytes

in this region express mRNA for PACAP receptors. Having found PACAP mRNA in NAc afferents and PACAP receptor mRNA in NAc astrocytes, we then investigated whether PACAP application alters AMPAR-mediated EPSCs in NAc efferents, similar to what has been observed in other brain regions (Kopp et al., 2001; Costa et al., 2009; Gardoni et al., 2012; Toda and Huganir, 2015). Having found that PACAP depresses AMPAR-EPSCs in NAc efferents projecting to the substantia nigra, we determined whether this effect requires Sxc regulation. In support of this idea, PACAP did not alter synaptic transmission in NAc slices obtained from loss of Sxc-function transgenic rats (MSxc rats). Lastly, we examined the relevance of these findings to addiction by assessing the impact of PACAP microinjection into the NAc on cocaine-primed reinstatement. Intra-NAc PACAP blocked cocaine reinstatement in wild-type but not MSxc rats. Collectively, these data are the first to establish that PACAP signaling is a key component of excitatory signaling in the NAc.

Material and Methods

Animals and Materials: These experiments utilized Sprague Dawley rats purchased from Envigo (Indianapolis, IN) and Sprague Dawley wild-type (WT) and transgenic mutants obtained from internal colonies, including Sxc loss of function mutants (MSxc) and eGFP knock-in (GFAP-lck-eGFP) rats. Experimental procedures were approved by the Marquette University Institutional Animal Care and Use Committee. The primary materials included fetal bovine serum and horse serum (Atlanta Biologicals, Lawrenceville, GA), ^{14}C-cystine (PerkinElmer, Waltham, MA), PACAP1-38 (California Peptide Research, Napa, CA), and PACAP6-38 (Anaspec, Fremont, CA).

PACAP1-38 (PACAP) is the endogenous full-length peptide whereas PACAP6-38 is a truncated version of PACAP and inhibits PACAP receptors (Miyata et al., 1989; Robberecht et al., 1992; Arimura, 2007; Vaudry et al., 2009). Picrotoxin and all other common chemicals were obtained from Sigma-Aldrich (St Louis, MO). Tetrodotoxin (TTX) was obtained from Tocris Bioscience (Ellisville, MO).

Creation of GFAP-lck-eGFP Rats: GFAP-lck-eGFP rats were generated using *Sleeping Beauty* (SB) transpositional transgenesis (Katter et al., 2013; Ivics et al., 2014). Briefly, a plasmid harboring a SB transposon transgene consisting of the GFAP promoter (Glial Fibrillary Acidic Protein), Lck-eGFP cDNA, and rabbit beta globin polyadenylation signal, was injected into fertilized embryos with an *in vitro* transcribed source of the SB100X transposase. A transgenic animal was identified harboring a single copy of the transgene inserted on chromosome 13, near 97.97Mb (sequence tag: TAC CTC TGT GAA GAC CCT CAG AGA GCT GTA ATT GTT CAT CCA AAT TTG AAG ACT TTG). A colony was established by back crossing to the parental Crl:SD strain and a fixed transgenic line was established by intercrossing. Genomic PCR primers GFAP04_forward 5' –TTT TCC TGA ATT TAT GTT TGT GCA G -3', GFAP04_reverse 5'- TCA TTC TTA GCT GTT TGA CAT CTG TCC -3', and a transposon specific primer 5'-GAC TTG TGT CAT GCA CAA AGT AGA TGT CC - 3' were used to determine zygosity.

Surgeries: All surgeries were conducted with rats under ketamine HCl (100 mg/kg, i.p.; Henry Schein, Dublin, OH) and xylazine (2 mg/kg, i.p.; Lloyd Laboratories, Shenandoah, IA) induced anesthesia.

Microinjection of Retrograde Tracers: In order to label NAc afferents, red beads (fluorescent latex microspheres that contain rhodamine; Lumafluor Inc., Durham, NC; used for FACS) or Cholera Toxin Subunit B (CTB; Thermo Fisher Scientific, Waltham, MA; used for immunostaining and *in situ* hybridization) were injected into the NAc. In order to label NAc efferents, red beads were injected into either the ventral pallidum (VP) or substantia nigra (SN) to label NAc-VP or NAc-SN medium spiny neurons (MSNs), respectively. In each case, guide cannula (C315G, 26GA, 11mm; Plastic One) were surgically implanted 2mm above the target structure with the coordinates (skull leveled, with Bregma as reference) derived from Paxinos and Watson (2007): Substantia Nigra (SN), AP -5.0, ML +3.0mm, DV -6.1mm, 6^{o} angle from midline; Ventral Pallidum (VP), AP -0.2mm, ML +3.2mm, DV -5.8mm, 6^{o} angle from midline; Nucleus Accumbens (NAc) core, AP +1.7mm, ML +2.4mm, DV -5.3 mm, 6^{o} angle from midline. Microinjectors (C315I-SPC, 33GA, with 2mm projection; Plastic One) were inserted in the cannula. Tracer was then microinjected with 2 µL Hamilton syringes at a rate of 50nL/min, to reach a target volume of 300 nL/hemisphere for SN and VP, and 500 nL/hemisphere for the NAc. For the purpose of a thorough perfusion of the beads, microinjectors remained unremoved after microinjection for 10min and were then slowly pulled out together with cannula. Openings on the skull for guide cannula were then sealed with bone wax before scalp being sutured.

Implantation of jugular catheters: Rats included in self-administration studies were implanted with indwelling catheters. In brief, a custom polyurethane catheter (Access Technologies, Skokie, IL) was implanted such that it entered the superior vena cava 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, Roanoke, VA) attached with dental acrylic to a piece of polypropylene monofilament mesh, exited 2 cm posterior to the scapulae. Throughout the experiment, catheters were filled daily with a heparin solution (83 i.u./ml; Elkins-Sinn, Cherry Hill, NJ) and capped when disconnected from the leash/delivery line assembly.

Implantation of Intracranial Guide Cannula: Some rats received indwelling bilateral guide cannulas (26 gauge, 14 mm; Plastics One) targeting the NAc core using the following coordinates derived from Paxinos and Watson (2007): AP +1.7mm, ML +2.4mm, DV -5.3mm, 6^{o} angle from vertical (skull leveled, with Bregma as reference). Dummy internals were inserted in the cannula to prevent blocking. After surgeries, rats were given at least 7 days to recover before testing. During this time, rats were provided chewable Rimadyl tablets (first three days, Bio-Serv, Flemington, NJ) and injected daily with sterile cefazolin antibiotic solution (15 mg, i.v.; Sagent Pharmaceuticals, Schaumburg, IL).

Cell Dissociation: The NAc or prefrontal cortex (PFC) tissues were freshly dissected from male GFAP-lck-eGFP or WT Sprague Dawley rats and placed immediately into ice-cold Hanks ´Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Sigma-Aldrich, St Louis, MO). Tissues were further minced before enzymatic digestion to obtain single cell suspension with Neural Tissue Dissociation Kit - P (Miltenyi Biotec, San Diego, CA), following manufacturer's protocol. 1% BSA, 1mM EDTA and 12.5 U/ml DNase DNase I (Sigma, Cat. DN25) were added to single cell suspension to reduce cell clustering. Cells were incubated with 3 µM Calcein Violet 450 AM Viability Dye (eBioscience, San Diego, CA) on ice for 10min to stain live cells.

Fluorescence-Activated Cell Sorting (FACS): FACS analyses were performed using FACSDiva software, 6.1.3, (BD Biosciences). Fluorescence Minus One (FMO) control samples derived from store-bought Sprague Dawley rats were first analyzed to set primary gates to exclude double cells, dead cells and debris. An aliquot of cell suspension was extracted as unsorted total population prior to cell sorting. Target cells were collected from a population with high levels of calcein violet staining, indicating live cells. GFP+ and GFP- (see Figure 4.1), or rhodamine + and rhodamine - (see Figure 4.8) cell populations were isolated through FACS based on the intensity of the respective fluorophore (e.g. GFP, rhodamine). Flow rate was carefully adjusted to increase purity.

RNA Extraction and cDNA Construction: Total RNA from sorted cells was isolated with PicoPure RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's protocol. On column DNase treatment was applied to all samples to remove potential genomic DNA contamination with the RNase-Free DNase Set (Qiagen; Germantown, MD). RNA quantity and quality were assessed on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Chips. Only those samples with higher quality (e.g RIN > 7.0) were used for downstream procedures.

Total RNA from brain tissues or cell cultures was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. DNase treatment was applied to all RNA samples to remove potential genomic DNA contamination with a DNA-free kit (Life Technologies, Carlsbad, CA). Assessment of RNA purity and quantity was performed on a NanoVue Plus Spectrophotometer (GE life sciences, Pittsburg, PA).

cDNA was constructed from total RNA using the Reverse Transcription System (Promega, Madison, WI) with oligo(dT) primers following the manufacturer's protocol.

Real-Time Quantitative Polymerase Chain Reaction (qPCR): qPCR was performed using a StepOne Real-Time PCR System (Applied Biosystems; Carlsbad, CA) and PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersberg, MD). Relative quantification of target gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). Primer sequences were as follows: xCT (catalytic subunit of Sxc) forward - 5' AGG GCA TAC TCC AGA ACA CG 3'; xCT reverse - 5' ATG CTC GTA CCC AAT TCA GC 3'; PAC1R forward - 5' TGC CTG TGG CTA TTG CTA TG 3'; PAC1R reverse - 5' TTT AGT CCC ATC AGG TCG TTG 3'; GFAP forward - 5' GCA GGT GAG GAA GAA ATG GA 3'; GFAP reverse - 5' TAC GAT GTC CTG GGA AAA GG 3'; NeuN forward - 5' ATC ATA CCA TCG GCC CCA CA 3'; NeuN reverse - 5' GTG AAG CGG CTG TAC CCT CC 3'. GAPDH forward - 5' CTC CCA TTC TTC CAC CTT TGA 3'; GAPDH reverse - 5' ATG TAG GCC ATG AGG TCC AC 3'. Primers were designed using the online primer design tool Primer3 (http://biotools.umassmed.edu/bioapps/ primer3_www.cgi). Another online software Primer-Blast (https://www.ncbi.nlm.nih. gov/tools/primer-blast/) was used to confirm the target-specificity of the primers. A single product from amplification was confirmed by melt curve analysis. Amplification efficiency of all genes was determined to be approximately 95%.

Creation of Sxc loss of function mutants (MSxc): Zing-finger nucleases (ZFNs) were procured from Sigma Aldrich to target xCT-encoding gene SLC7A11. ZFNs were

introduced to SD rat embryos at the one-cell stage by pronuclear microinjection of *in vitro*-transcribed ZFN mRNAs, to create whole animal disruption of SLC7A11 in a single step. An xCT mutant was identified with a 39-bp deletion in exon 2 of SLC7A11 gene, which predictably leads to the missing of 13 amino acids (see Figure 4.3A) that correspond to the majority of the 3rd transmembrane domain of the xCT protein.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis: PCR was performed with GoTaq Green Master Mix (Promega, Madison, WI) on a thermocycler with parameters as follows. Initial denaturation (3min at 95℃), Amplification (35 cycles of the following: 95℃ for 30s, 60℃ for 45s, 72℃ for 45s), and Extension (72℃ for 10min). PCR products were then examined with electrophoresis on an agarose gel (1.8%). Sequences of primers used to target the mutation sites in xCT KO rats were as follows, xCT M forward - 5’ TTT GGA GCC CTG TCT TAT GC 3’; xCT M reverse - 5’ ACC CAG ACT CGA ACA AAA GC 3’. Other primers used for PCR included GFAP forward - 5’ GCA GGT GAG GAA GAA ATG GA 3’; GFAP reverse - 5’ TAC GAT GTC CTG GGA AAA GG 3’; NeuN forward - 5’ ATC ATA CCA TCG GCC CCA CA 3’; NeuN reverse - 5’ GTG AAG CGG CTG TAC CCT CC 3’; PACAP forward-5’ AAC CCG CTG CAA GAC TTC TA 3’; PACAP reverse- 5’ CTT TGC GGT AGG CTT CGT TA. 100bp DNA Ladder (Promega, Madison, WI) was used to locate target PCR products.

Western Blotting: NAc tissues of WT or MSxc rats were mechanically homogenized in buffer containing 320mM sucrose and 10mM Tris-HCl, supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). 10 μg of total protein was resolved in a 7.5% polyacrylamide SDS-PAGE gel followed by

wet transfer to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with Odyssey Blocking Buffer (PBS) (LI-COR, Lincoln, NE) for one hour prior to an overnight treatment with a mouse anti-GluA1 antibody (1:1000, Millipore), a rabbit anti-pT840 antibody (1:3333, Abcam), a chicken anti-GAPDH antibody (1:10000, Millipore), or a custom rabbit anti-xCT antibody that was generously donated to us by Dr. Jeffrey Rothstein at John Hopkins School of Medicine. Next, membranes were rinsed and incubated with IRDye 680 goat anti-rabbit (1:15000, LI-COR) or IRDye 800 donkey anti-chicken (1:20000, LI-COR) secondary antibodies for one hour. Membranes were rinsed again prior to imaging on an Odyssey Fc Imaging System (LI-COR).

***Ex vivo* ^{14}C-cystine Uptake Assays:** The assessment of system x_c- (Sxc) activity is often achieved by measuring intracellular uptake of radiolabeled cystine, since this is primarily dependent on Sxc (Liu et al., 2009; Liu et al., 2014a; Resch et al., 2014b; Albano et al., 2015). The NAc tissue punches (1mm thick and 1mm in diameter) were freshly dissected and placed immediately into artificial cerebrospinal fluid that was constantly infused with 95% O_2:5% CO_2. Following a 20-minute equilibration period during which the buffer was warmed to 37^{o}C, ^{14}C-cystine and DL-threo-β-hydroxyaspartic acid (TBOA) were added to the media to reach a final concentration of 1μM and 10 μM, respectively. This concentration of cystine was used since it is similar to extracellular cystine concentrations in the brain (Baker et al., 2003). TBOA was added to the culture media to prevent ^{14}C-cystine uptake by sodium-dependent glutamate transporters. Some conditions also involved the addition of the Sxc inhibitor sulfasalazine (SSZ, 300 μM) to block Sxc-mediated cystine uptake. Immediately following 20-min exposure to^{14}C-cystine, tissues were washed three times with ice-cold 1xPBS and then

solubilized with 1N NaOH. One aliquot of tissue lysate was used to determine protein concentration with BCA protein assay and another aliquot was used for ^{14}C-cystine uptake determination. ^{14}C-cystine uptake was normalized to protein concentration and data were presented as CPM/μg of protein.

Cell Culture Procedures: Purified striatal astrocyte cultures were prepared from post-natal days 3-4 rat pups. In brief, striatal cells were dissociated and then suspended in Neurobasal A media (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, and 1% Glutamax (Thermo Fisher Scientific, Waltham, MA). Cells were initially grown in 25cm^2 or 75cm^2 cell culture flasks that were pre-coated with poly-D-lysine (10mg/L) and laminin (0.4mg/L). Once confluent, cells were then subjected to prolonged, orbital shaking (250 rpm for 16-18 hours at 37^{o}C), which has been used to yield purified astrocyte cultures (McCarthy and de Vellis, 1980; Schildge et al., 2013). Purified astrocytes were then plated on 24-well plates, and refreshed with 70% new culture media every 2-3 days prior to experiments. All cell cultures were maintained in humidified 5% CO_2 incubators at 37^{o}C.

Glutamate Release Assay and Glutamate HPLC: Purified striatal astrocytes (DIV14) were incubated with PACAP at designated concentrations for 30 minutes at 37^{o}C in a Na^+ free buffer containing 116mM Choline Chloride, 13.4 mM $MgSO_4$, 1.68 mM KH_2PO_4, 2.34 mM $CaCl_2$, 5.49 mM Dextrose, 11.9 mM HEPES, 10 μM cystine and 0.2% Choline bicarbonate. Buffer pH was titrated to 7.4 with NaOH. After incubation, 100 μL media was extracted for HPLC analysis. Cells were solubilized in 0.5% sodium dodecyl sulfate (SDS). One aliquot of cell lysate was used for protein quantification using the bicinchoninic acid (BCA) assay.

Media collected in glutamate release assays were analyzed for glutamate content via fluorescence detection on an isocratic Shimadzu HPLC system. A 10µL sample underwent pre-column derivatization with ortho-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol. Samples were resolved with a Kinetex XB C-18 (50x4.6mm, 2.6µm; Phenomenex, Torrence, CA) and 100mM Na_2HPO_4, 0.1mM Ethylenediaminetetraacetic acid (EDTA), 10% Acetonitrile, pH 6.04 mobile phase. Peak areas were quantitated in Shimadzu VP client software compared to known standards. Glutamate content for each sample was normalized to protein concentration.

Brain slice preparation: Rats were anesthetized by isoflurane inhalation and perfused through the aorta with a cold sucrose-based solution (4–6 °C) containing 78 mM NaCl, 68 mM sucrose, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 25 mM glucose. The rat brain was trimmed and embedded in low-melting-point agarose, and coronal striatal slices (200-250 µm thick) were cut in the sucrose-based solution (4-6 °C) using a vibrating slicer (Leica VT1200s, Nussloch, Germany). The slices were incubated in the sucrose-based solution at room temperature for 30-40 min. Then, the slices were transferred and allowed to recover for at least 1 hour in the artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose. All solutions were saturated with 95% O_2 and 5% CO_2.

Electrophysiology: <u>AMPAR-mediated EPSC</u> Male Sprague Dawley rats (300-350g) received a bilateral microinjection of red beads into SN or VP. After 3-5 days of recovery, rats were anesthetized with isoflurane and decapitated. Striatal slices were prepared as previously described (Liu et al., 2014b). Whole-cell voltage-clamp

recordings were made from medium spiny neurons (MSNs) in the core of the NAc. MSNs that were retrogradely labeled with red beads were selected for recording. AMPAR-EPSCs were evoked by a bipolar tungsten stimulation electrode that was placed ~300 μm away from the recording pipette. The MSNs were voltage-clamped at -80 mV, close to the resting membrane potential of these neurons. PACAP or PACAP6-38 was administered through bath application.

Cocaine Self-Administration Training: Self-administration occurred in operant chambers (ENV-008CT; MEDAssociates, St. Albans, VT) housed in sound-attenuating cubicles (ENV- 016M; MED-Associates) and equipped with two retractable levers, two stimulus lights, and a water bottle. At least 7 d after surgery, rats were food restricted for 18 h with water available *ad libitum*. Rats were then placed into the operant 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 100 food rewards in a session, which typically occurred after the first session. During the acquisition phase of the experiment, all rats underwent drug self-administration training during daily 2 h sessions in which operant responses on the active lever were reinforced with an infusion of saline or cocaine (0.5 mg/kg/200 μL, i.v.; National Institute on Drug Abuse, Bethesda, MD) 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.

Once rats met the acquisition criteria, they were advanced to maintenance sessions in which saline or cocaine was self-administered under long-access conditions (1.0 mg/kg/200 µL, i.v.; 6 h/d for 12 d).

Extinction Training: After completing maintenance self-administration sessions, rats remained in their home cages for 7 d before extinction training. A seven-day delay was used to ensure an adequate drug-free period before reinstatement, even in rats that quickly extinguished responding. Extinction training involved placing rats into the operant chambers for 2 h/d as described above in the self-administration section except each active lever press now resulted in an infusion of saline. This continued until the mean number of lever presses was no more than 15 responses across two sessions, at which point rats were tested for cocaine-primed reinstatement.

Microinjection and Reinstatement Testing: Once rats reached extinction criteria, mock microinjections were conducted to familiarize rats with the procedure approximately two days prior to reinstatement test day. The reinstatement test was identical to each extinction session except rats were injected with cocaine (10 mg/kg, IP). Prior to the cocaine injection, microinjectors were inserted into indwelling guide cannula, and rats received infusion of vehicle [0.9% NaCl], PACAP [100µM] or [200µM], or PACAP6-38 [1000µM] at a rate of 0.125 µL/min for four minutes. Six minutes after microinjection, rats were given an injection of cocaine (10 mg/kg, IP), and then immediately placed in the self-administration chamber for reinstatement test, behavior was recorded for 120 min.

Histology: Rats that received implantation of indwelling guide cannula were anesthetized with CO_2, and the brains removed and imbedded in OCT compound.

Coronal sections (30 μm) were cut on a cryostat and stained with cresyl violet to verify probe placements.

CTB Immunostaining and PACAP *In Situ* Hybridization: One week after microinjecting CTB into the NAc, brains were collected via rapid decapitation and flash frozen in OCT with a dry ice/ethanol bath. Brains were kept at -80^{o}C before being cut on a cryostat into coronal sections (12 μm thick) which were thaw-mounted onto electrostatically clean slides. The brain sections were stored at -80 ℃ until *in situ* hybridization. Prior to overnight hybridization, the brain sections were post-fixed in 4% PFA, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0) and acetylated in triethanolamine containing 0.25% acetic anhydride. Sense and antisense digoxigenin conjugated riboprobes specific for PACAP were diluted (1:100) in a hybridization solution (Amersco; Solon, OH) containing 1/4th riboprobe volume of tRNA (4 μl riboprobe/1 μl tRNA). The probe cocktail was incubated for 10 minutes at 65 ℃ and then applied to the sections (100 μl/slide). Once the probe was applied, the slides were coverslipped and hybridized overnight at 60 ℃. Approximately 14-16 hours later, the slides were treated with an RNase A solution, stringently washed in 0.1x SSC at 65 ℃ and then incubated overnight at 4 ℃ with an antibody against DIG conjugated to a horseradish peroxidase (HRP; Roche; Indianapolis, IN). The DIG conjugated PACAP riboprobes were visualized using a TSA-plus fluorophore system with fluorescein (Perkin Elmer; Waltham, MA). Following the *in situ* hybridization procedure, slides were then incubated overnight in an antibody against CTB (Goat anti-CTB; List Biological Laboratories Inc.; Campbell, CA). This primary antibody was then visualized using a donkey anti-goat AlexaFluor 594 conjugated secondary antibody (Life Technologies;

Grand Island, NY). Images were then captured using fluorescent microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

Statistics: Data are presented as the mean ± SEM. The data of mEPSCs were analyzed using Mini-analysis (Synaptosft, Decatur, GA). The frequency and amplitude of mEPSCs were calculated for 3-5 min of recordings. The analysis of mEPSCs was performed with cumulative probability plots (Van der Kloot, 1991). All other statistical analyses were performed using SPSS Statistics (Version 24, IBM; Armonk, New York). Analysis of variance (ANOVA) was used when comparing data sets that included more than two groups. Bonferroni tests were used for subsequent *post hoc* analyses of significant effects involving more than two groups. Student's t tests were used when comparing results from only two groups. In all instances, statistical significance was designated as $p < 0.05$.

Results

Experiment 1: xCT and PAC1R mRNAs are enriched in adult NAc astrocytes. The purpose of this experiment was to determine whether PACAP receptors are expressed in NAc astrocytes isolated from adult tissues. In order to do this, we first characterized astrocyte labelling in GFAP:Lck-eGFP rats. To verify this, eGFP expression produced by the transgene was compared with GFAP-IHC (immunohistochemistry) in the same tissue. Figure 4.1B-D illustrates that eGFP provides a more complete labelling of astrocytic processes in GFAP+ cells. To further verify that the eGFP+ cells were astrocytes, we used FACS (fluorescence-activated cell sorting) to

isolate these cells from NAc tissue punches obtained from adult (i.e., aged 3-4 months) GFAP:Lck-eGFP transgenic rats (Figure 4.1E-J).

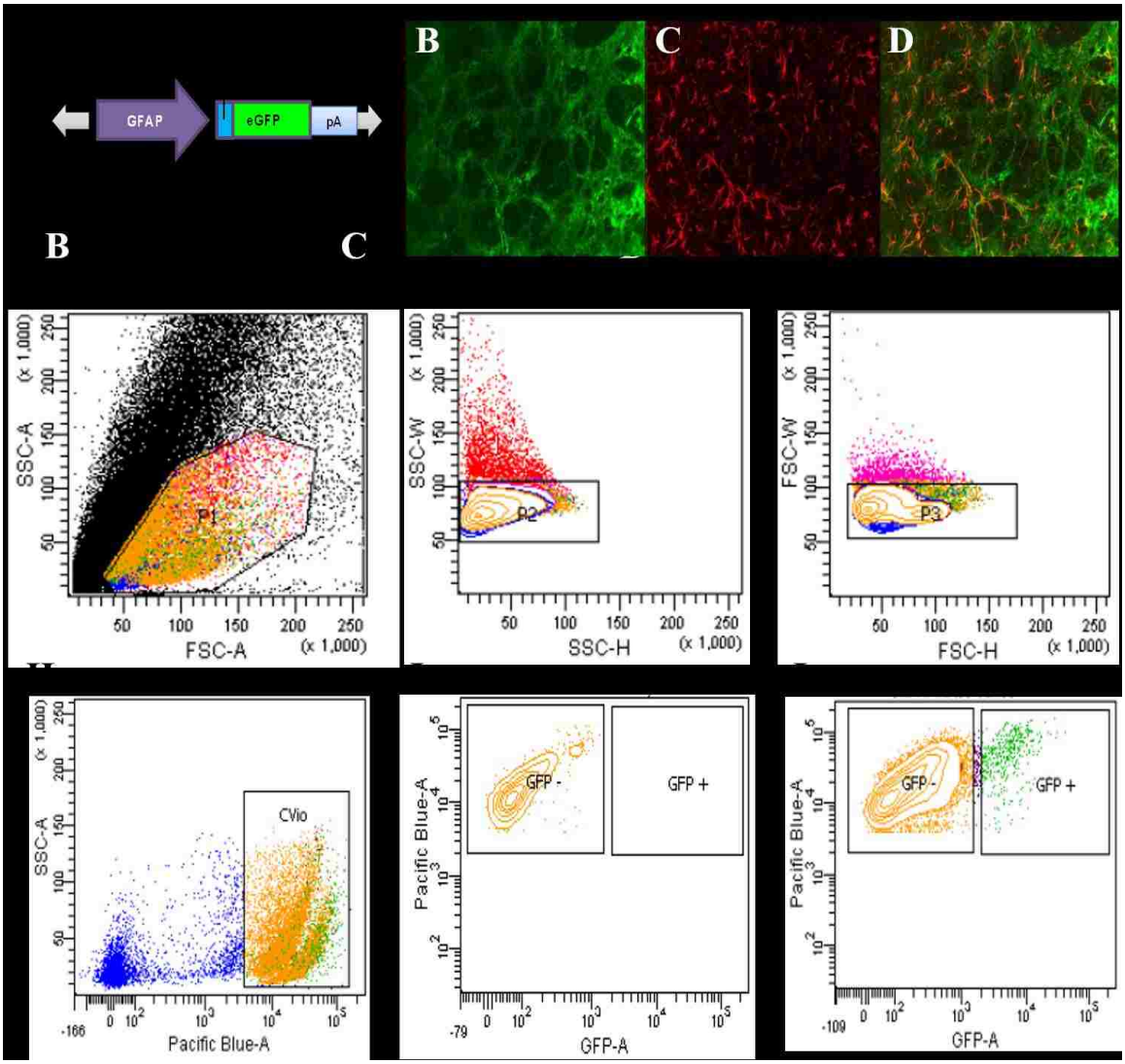

Figure 4.1. Isolation of adult NAc astrocytes from GFAP:Lck-eGFP transgenic rats with fluorescence-activated cell sorting. (A) Schematic diagram of the plasmid harboring a Sleeping Beauty transposon transgene consisting of the glial fibrillary acidic protein (GFAP) promoter, cDNA for a membrane-targeted form of enhanced green fluorescent protein (Lck-eGFP) (Benediktsson et al., 2005), and rabbit beta globin polyadenylation signal (pA). (B-D) Visualizing eGFP in GFAP:Lck-eGFP transgenic rat permits a more complete labelling of astrocytes (B) relative to GFAP immunoreactivity of the same tissue (C), see merged images (D). (E-J) The gating parameters of the flow cytometer were utilized to identify/isolate cellular debris (E; forward scatter area (FCS-A)/side scatter area (SSC-A), cell doublets (F: side scatter height (SSC-H)/side scatter area (SSC-A) and G: forward-scatter height/forward scatter width), viable cells (H; calcein violet/SSC-A), background green fluorescent protein in negative control tissue (I; tissue obtained from WT rats), and transgene-induced fluorescence above background (J: tissue obtained from GFAP:Lck-eGFP transgenic rats).

Next, we used PCR to compare the mRNA expression of astrocyte-enriched genes (i.e., GFAP, xCT) and neuron-enriched genes (NeuN). When including the three sample types (total or unsorted, GFP+, and GFP-), one-way ANOVAs revealed significant main effects of sample type for the expression of NeuN [Figure 4.2A; $F(2,9)=27.798$, $p < 0.001$], GFAP [Figure 4.2B; $F(2,9)=16.829$, $p = 0.001$], and xCT [Figure 4.2C; $F(2,9)=11.209$, $p < 0.01$]. *Post hoc* tests revealed that GPF+ cells contained significantly higher mRNA levels of the positive control genes GFAP and xCT (Tukey HSD, $p < 0.05$) and did not express the negative control gene NeuN (Figure 4.2C), indicating that this astrocyte-enriched sample did not contain neurons.

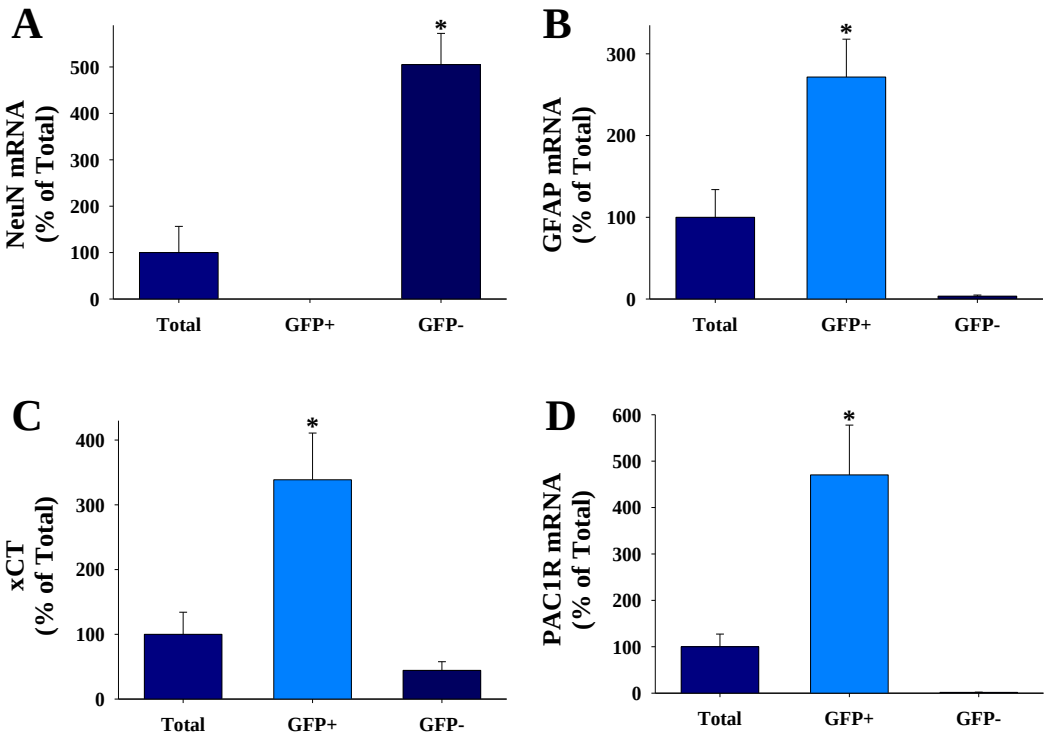

Figure 4.2. xCT and PAC1R mRNAs are highly expressed in rat NAc astrocytes. Data depict Mean ± standard error of means (SEM) mRNA levels of NeuN (A), GFAP (B), xCT (C), and PAC1R (D) in unsorted (total) cell populations, GPF+, and GFP- isolates (N= 4/gene/population) from NAc tissues. * indicates a significant difference relative to unsorted total population, Tukey HSD, $p < 0.05$.

Next, we examined mRNA expression of the test gene PAC1R in the three tissue samples (total or unsorted, GFP+, and GFP-). A one-way ANOVA yielded a main effect of sample type on the PAC1R mRNA expression [Figure 4.2D, $F(2,9)=14.993$, $p = 0.001$] with *post hoc* analyses revealing enrichment in the GFP+ cells (Tukey HSD, $p < 0.05$).

Experiment 2: PACAP regulates Sxc mediated glutamate release. Given that NAc astrocytes express the receptor needed to encode PACAP signaling, we then examined whether this neuropeptide can regulate glutamate release by Sxc. Note, in Chapter III, our measure of interest was primarily cystine uptake. Thus, we sought to confirm that PACAP would promote glutamate release from striatal astrocytes. To do this, we used loss of function Sxc mutant (MSxc) rats. Zinc-finger nucleases were used to mutate the gene (Slc7A11) that encodes the protein (xCT) containing the binding sites for the transported substrates (i.e., cystine, glutamate). The mutation was identified as a 39-bp deletion in exon 2 of SLC7A11, which predictably leads to the loss of 13 amino acids (Figure 4.3A) that corresponds to the majority of the 3rd transmembrane domain of the xCT protein. For further validation of MSxc rats, we examined the expression of xCT mRNA and protein, and Sxc function in striatal tissues (Figure 4.3B-D). Using primers that partially corresponded to the mutation region, we confirmed the absence of intact xCT mRNA in the mutants (Figure 4.3B). Consistent with this observation, MSxc rats did not display xCT protein as assessed using western blotting (Figure 4.3C). To evaluate the functional consequence of the mutation, we compared ^{14}C-cystine uptake in NAc tissue punches in the presence or absence of an Sxc inhibitor sulfasalazine (SSZ). A univariate ANOVA with genotype and SSZ concentration as between-subjects factors produced a significant interaction [Figure 4.3D; $F(1,15)=13.098$, $p < 0.01$]. Further

analyses of the data revealed that SSZ significantly reduced uptake levels in WT (T-test, $p < 0.01$) but not in MSxc tissues, suggesting a lack of Sxc activity in the mutants.

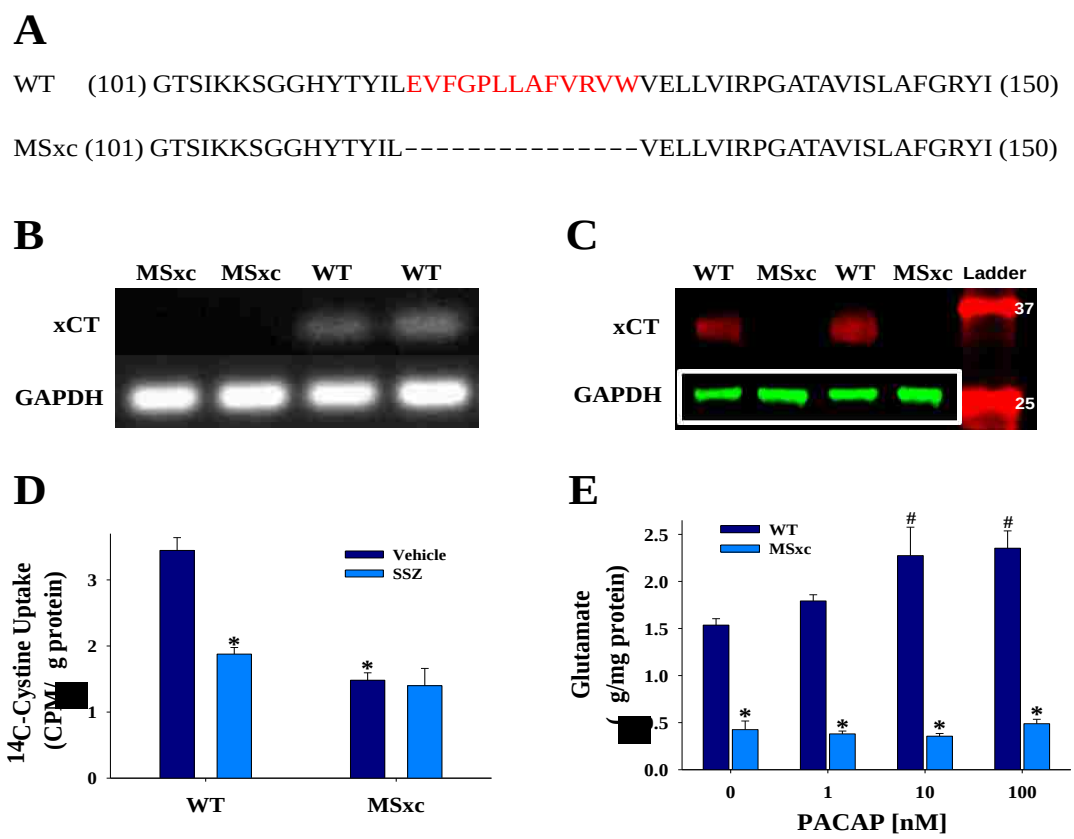

Figure 4.3. PACAP regulates Sxc mediated glutamate release. (A) Amino acid sequence (101-150) of xCT in MSxc and WT rats. Zinc-finger nucleases (ZFNs) specific for exon 2 of the rat SLC7A11 gene (encoding xCT, the catalytic subunit of Sxc) were used to create Sxc mutants; the sequence eliminated by the mutation is denoted by the dotted lines. (B) Gel electrophoresis results showing the lack of intact xCT mRNA in MSxc striatum. Reverse transcription and PCR were used to validate the elimination of xCT mRNA sequence corresponding to AAs 116-128 as depicted in panel A. (C) Western blot analysis was used to confirm a lack of xCT protein in MSxc NAc tissue punches. (D) Data depict the mean ± SEM levels of total (i.e., in the presence of vehicle) or non-Sxc-mediated (i.e. in the presence of the Sxc inhibitor sulfasalazine, SSZ; 300 µM) ^{14}C-cystine uptake in WT or MSxc striatal tissue punches (N=3-6/condition/genotype). * indicates a significant difference relative to WT tissues treated with vehicle, T-test, $p < 0.01$. (E) Data depict the mean ± SEM levels of extracellular glutamate levels in purified striatal astrocytes obtained from WT or MSxc rats after PACAP treatment (30 min at 0-100 nM; N=6/condition). * indicates a significant difference relative to WT with the corresponding PACAP treatment, T-test, $p < 0.001$; # indicates a significant difference relative to WT astrocytes treated with vehicle, Tukey HSD, $p < 0.05$.

Next we examined the impact of PACAP on Sxc-mediated glutamate release in WT or MSxc striatal astrocytes following PACAP application (30 min). A univariate ANOVA with genotype and PACAP concentration as between subjects factors produced a significant interaction [Figure 4.3E; $F(3,40)=4.058$, $p<0.05$]. Further analyses revealed significantly lower levels of glutamate in MSxc astrocytes compared to WT at each of the PACAP concentrations (T-test, $p<0.001$), consistent with the lack of Sxc activity in the mutants. A one-way ANOVA with PACAP concentration as a between-subjects factor revealed a PACAP dose-dependent increase of glutamate in WT [$F(3,20)=4.564$, $p<0.05$] but not in MSxc astrocytes, suggesting that PACAP potentially increases astrocyte Sxc-mediated glutamate release in rat striatum.

Experiment 3: PACAP attenuates AMPAR-mediated EPSCs in SN-projecting MSNs in the NAc. Having established that PACAP regulates astrocyte glutamate release mediated by Sxc, we then determined whether this neuropeptide regulates synaptic transmission in a manner that could contribute to behavior linked to this structure. To do this, we examined the impact of PACAP application on the activity of the two primary NAc efferents, NAc-SN and NAc-VP MSNs, each of which are linked to goal-directed behaviors (Kravitz and Kreitzer, 2012). We found that bath application of PACAP at 100 nM induced significant depression of AMPAR-EPSCs [$t(12) = 2.10$, $p < 0.05$] but failed to do so at 1 nM (Figure 4.4A) in SN-projecting NAc MSNs. In contrast, PACAP at 100 nM or 0.1 nM did not alter AMPAR-EPSCs (Figure 4.4B) in VP-projecting NAc MSNs.

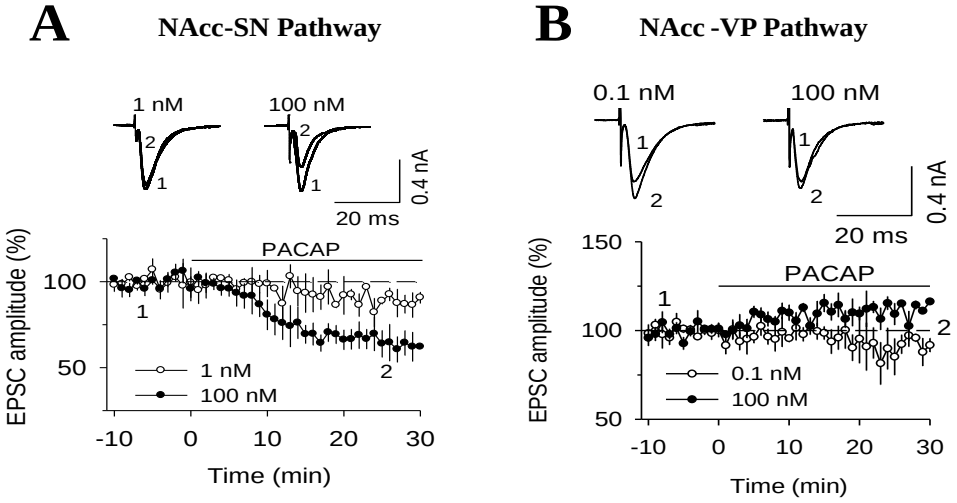

Figure 4.4. PACAP attenuates AMPAR-mediated EPSCs in SN-projecting NAc MSNs. (A) Data depict AMPAR-mediated EPSCs in retrogradely labeled NAc MSNs that project to the SN when incubated with PACAP at 1 nM (n = 7) or 100 nM (n = 8). 100 nM PACAP depressed AMPAR-mediated EPSCs. (B) Data depict AMPAR- mediated EPSCs in VP-projecting MSNs in the NAc when incubated with PACAP at 0.1 nM (n = 15) or 100 nM (n = 8).

Experiment 4: Characterization of PACAP-induced regulation of AMPAR-mediated EPSCs. To investigate the mechanisms mediating the actions of PACAP (100 nM), we first examined the involvement of PACAP receptors in PACAP-induced regulation of synaptic transmission in NAc tissue slices. When co-applied with the PACAP receptor inhibitor PACAP6-38, PACAP-induced depression of AMPAR-EPSCs in NAc-SN MSNs was abolished [Figure 4.5A; $t(13) = 3.19$, $p < 0.01$].

Next, we sought to test the hypothesis that PACAP regulates synaptic transmission via an Sxc-dependent mechanism. Support for this key hypothesis would provide a clear demonstration that PACAP functions as a molecular bridge linking astrocytes and neurons in the regulation of excitatory signaling-induced synaptic transmission in the NAc. We found that PACAP altered AMPAR-EPSCs in NAc tissue slices obtained from WT but failed to do so in MSxc rats [Figure 4.5B; $t(12) = 3.09$, $p <$

0.01]. This finding is consistent with the hypothesis that the neuropeptide PACAP integrates glutamate release from astrocytes to regulate synaptic transmission in the NAc.

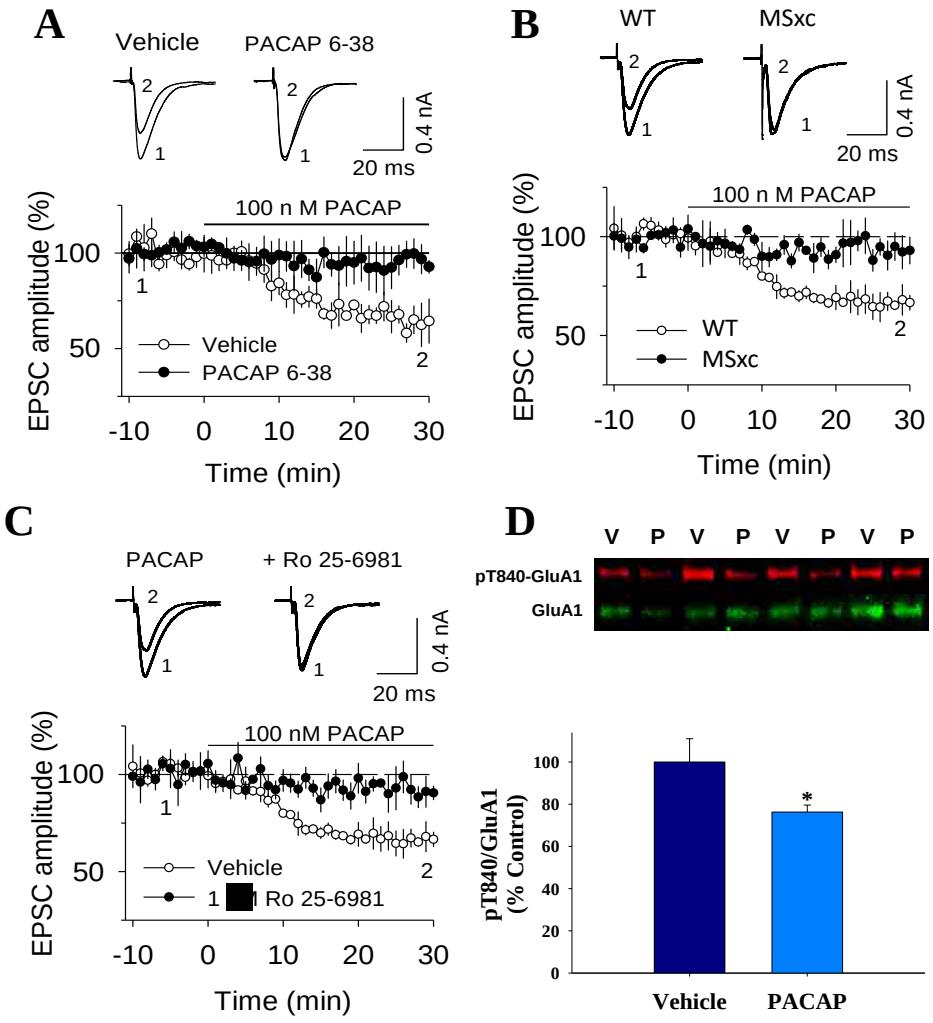

Figure 4.5. PACAP-induced regulation of AMPAR-mediated EPSCs in SN-projecting MSNs involves PACAP receptors, Sxc, NMDARs and possibly phosphorylation of GluA1-T840. (A-C) Data depict the effects of PACAP (100 nM) on AMPAR-mediated EPSCs in SN-projecting MSNs in the NAc, with or without the presence of PACAP receptor antagonist PACAP 6-38 (A; N= 7-8/treatment), in WT or MSxc rats (B; N=7/genotype), or with or without the presence of GluN2B-selective NMDAR antagonist Ro 25-6981 (C; 1μM, N= 6-7/treatment), (D) Data depicts the effect of PACAP microinjection (100 pmol; N=7-8/group) into the NAc on the phosphorylation of GluA1-T840. Upper panel: representative western blotting results; lower panel: data depicts the phosphorylation levels (mean+ SEM) of GluA1-T840 in vehicle and PACAP groups (normalized to vehicle group; N=7-8/group). V, vehicle; P, PACAP; pT840, phosphorylated T840. * indicates a significant difference relative to vehicle group, T-test, $p < 0.05$.

Prior work has implicated GluN2B-containing NMDARs, which are high-affinity glutamate receptors that can be tonically activated by astrocytes and expressed extra-synaptically (Barria and Malinow, 2002; Yoshii et al., 2003; van Zundert et al., 2004; Herman and Jahr, 2007; Le Meur et al., 2007; Hamilton and Attwell, 2010; Petralia et al., 2010; Gladding and Raymond, 2011; Paoletti, 2011; Povysheva and Johnson, 2012; Paoletti et al., 2013), in PACAP-induced regulation of synaptic transmission in hippocampal neurons (Toda and Huganir, 2015). Hence, we sought to determine if these receptors also contribute to PACAP-induced regulation of synaptic transmission in the NAc. Interestingly, we found that co-application of the GluN2B-selective NMDAR antagonist Ro 25-6981 blocked PACAP-induced depression of AMPAR-EPSCs [Figure 4.5C; $t(11) = 2.97$, $p < 0.05$].

The negative regulation of AMPARs by extrasynaptic NMDARs is thought to involve dephosphorylation of the key subunit GluA1 at T840 (Delgado et al., 2007; Lee et al., 2007; Gray et al., 2014; Toda and Huganir, 2015). To assess if this may contribute to our above observations, we examined whether PACAP dephosphorylates NAc AMPARs at GluA1-T840. We found that one hour after PACAP microinjection (100 pmol/side) in the NAc, the phosphorylation of GluA1-T840 was significantly decreased [Figure 4.5D; $t(13) = 2.178$, $p < 0.01$].

Experiment 5: Increasing PACAP signaling in the NAc attenuates cocaine-induced reinstatement via the regulation of Sxc. Since either decreased activity of Sxc or activation of SN-projecting MSNs in the NAc are linked to the expression of drug-seeking behaviors (Baker et al., 2003; Madayag et al., 2007; Rogers et al., 2008; Moussawi et al., 2009; Amen et al., 2011; Moussawi et al., 2011; Kravitz et al., 2012;

Schmaal et al., 2012; Bossert et al., 2013; Macpherson et al., 2014), we predicted that increasing PACAP signaling in the NAc, which potentially restores Sxc function and suppresses the SN-projecting neurons, would reduce cocaine seeking. To test this, we first examined the impact of microinjecting PACAP in the NAc on cocaine-induced reinstatement in rats (Figure 4.6A). A one-way ANOVA with PACAP concentration as a between-subjects factor yielded a main effect on reinstatement responses [Figure 4.6A; $F(2,26)=8.814$, $p = 0.001$]. We then examined the impact of PACAP in WT and MSxc rats. Repeated measures with genotype as a between-subjects factor and microinjection treatment as a within-subjects factor revealed a main interaction [Figure 4.6B; $F(1,7)=6.945$, $p < 0.05$]. PACAP microinjection in the NAc decreased reinstatement levels in WT rats [$t(8) = 5.999$, $p < 0.001$) but failed to do so in MSxc subjects. Therefore, these data suggest that increasing PACAP signaling in the NAc decreased compulsive cocaine seeking by upregulating Sxc activity.

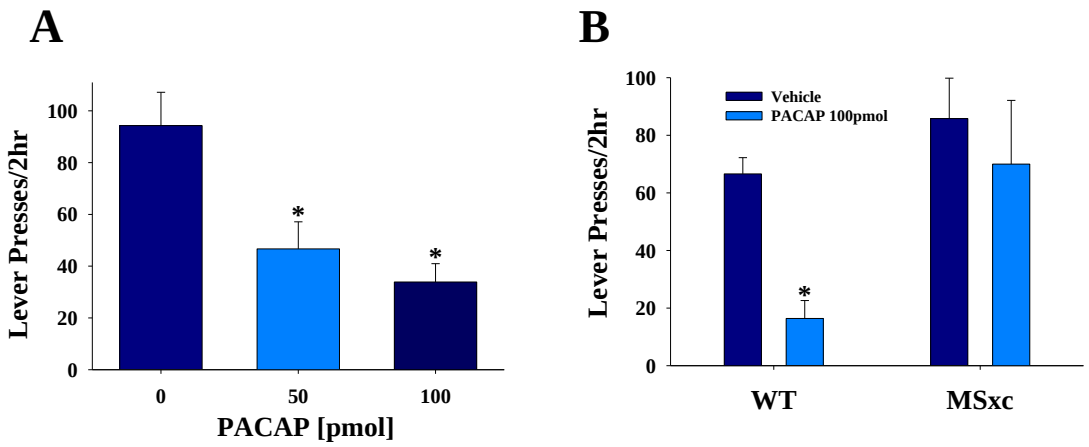

Figure 4.6. Increasing PACAP signaling in the NAc attenuates cocaine-induced reinstatement via the regulation of Sxc. Data depict lever responding during a two-hour session following intra-NAc PACAP microinjections (0-100 pmol) and a systemic injection of cocaine (10 mg/kg, i.p.) in commercially purchased WT rats (A; Envigo, Indianapolis, IN; N=6-13/condition) and in internally-generated WT and MSxc rats (B; N=4-5/condition). * indicates a significant difference relative to the vehicle group of WT rats, T-test, $p < 0.001$.

Experiment 6: PACAP is expressed in corticostriatal afferents to the NAc.

Having observed the importance of PACAP signaling in the NAc to synaptic transmission and motivated behavior, we were interested in investigating the sources of endogenous PACAP in this brain region. A number of studies implicate that PACAP is primarily, if not exclusively, expressed by glutamatergic neurons, and is co-transmitted with glutamate as a neurotransmitter (Masuo et al., 1992; Masuo et al., 1993; Koves et al., 1994; Hannibal et al., 2000; Hannibal, 2002a, b; Stumm et al., 2007; Engelund et al., 2010; Golombek and Rosenstein, 2010; Hu et al., 2011; Purrier et al., 2014). Given the importance of glutamatergic corticostriatal projections to the NAc to cocaine seeking, we hypothesized that these NAc afferents would express PACAP. To test this, we used two complementary approaches to determine if these cells express PACAP mRNA. In the first approach, we combined retrograde circuit tracing with *in situ* hybridization. Specifically, we microinjected retrograde tracer cholera toxin subunit B (CTB) in rat NAc (Figure 4.7A). *In situ* hybridization (ISH) shows expression of PACAP mRNA in the prelimbic PFC, particularly cortical layer V (Figure 4.7B), which has been suggested to possess a major neuronal population projecting to the NAc (Ding et al., 2001). Co-localization of the CTB and PACAP mRNA indicates that the majority of PFC neurons projecting to the NAc express PACAP (Figure 4.7C-E).

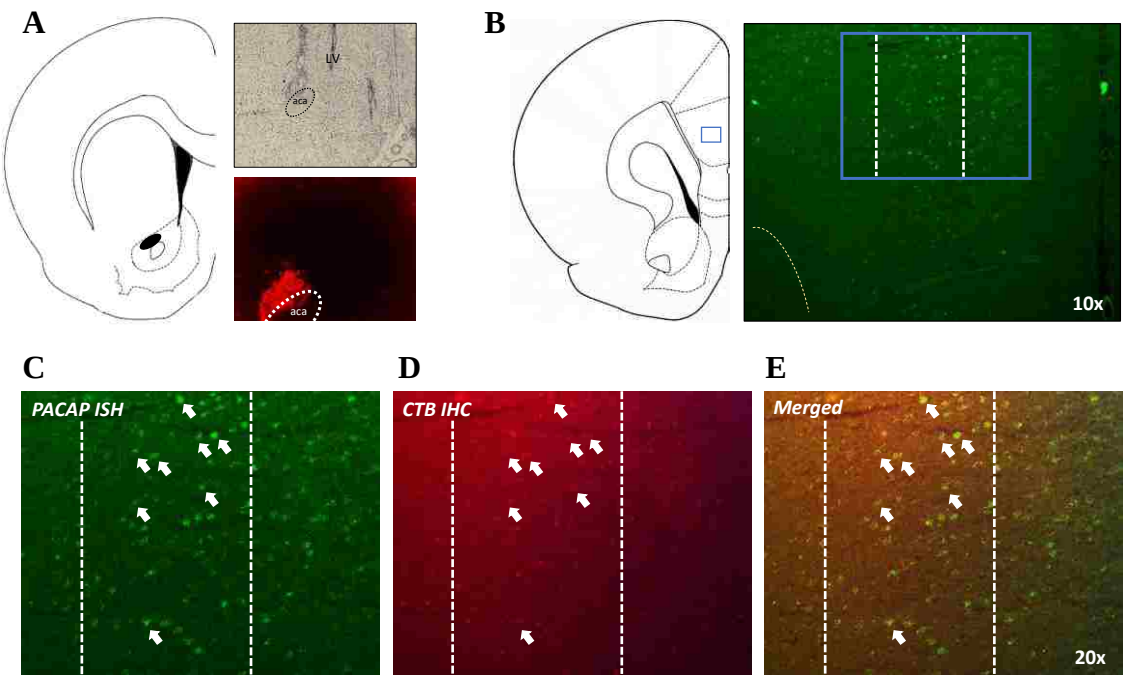

Figure 4.7. PACAP is expressed in NAc-projecting PFC neurons. (A) The retrograde tracer cholera toxin subunit B (CTB) was delivered to the NAc core. This was confirmed by visualizing cannula tracts (top right insert) and comparing the spread of CTB (bottom right insert) with the defined boundaries of the NAc core (left); LV, lateral ventricle. Aca, anterior part of anterior commissure. (B) A 10X photomicrograph illustrating PACAP mRNA in the prefrontal cortex as detected using *in situ* hybridization (ISH). The boxed area is further magnified to 20X in panel C-E. The vertical dotted lines define areas approximating layer V of the prelimbic cortex. (C-E) 20X photomicrographs illustrating PFC cells that express PACAP mRNA (C, ISH) and project to the NAc (D, CTB IHC). White arrows represent PFC-NAc projections that express PACAP.

The expression of PACAP in the PFC-to-NAc projecting neurons was confirmed using a second approach, in which we labelled corticostriatal projections by microinjecting a fluorescent retrograde tracer (rhodamine-containing micro-beads) into the NAc. The labeled cells were then isolated from the PFC using FACS (Figure 4.8A, B). Due to the limited efficiency of the experimental procedures and our aim for high circuit specificity, each sample/subject yielded a limited target population (several hundreds to several thousand positive events), yet, we were able to obtain high-quality RNA from such small samples, demonstrated by high RNA integrity numbers (RINs: 7-8.5 on a 1-10

scale; Figure 4.8C). Using PCR combined with gel electrophoresis, we observed the presence of PACAP mRNA in isolated PFC neurons projecting to the NAc (Figure 4.8D). Using this approach, we also detected PACAP mRNA in the basolateral amygdala (BLA) neurons projecting to the NAc. Collectively, these data provide the first demonstration that PACAP is expressed in the corticostriatal glutamatergic projections to the NAc.

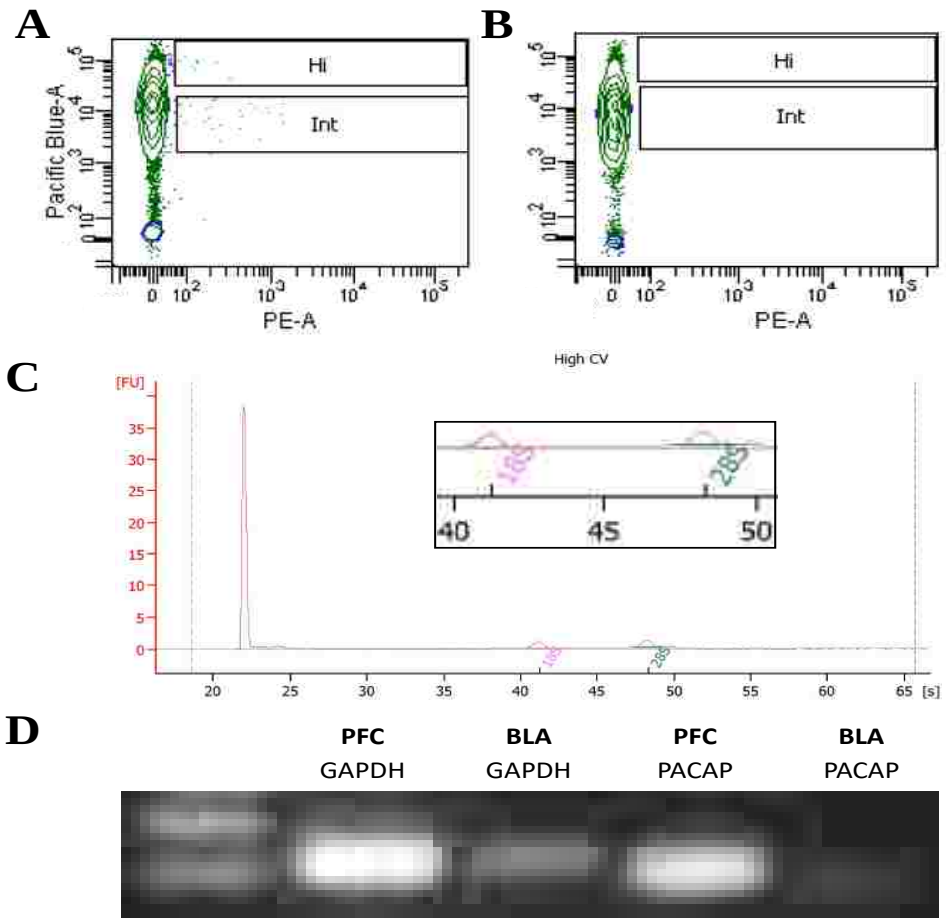

Figure 4.8. PACAP is expressed in PFC- and BLA-NAc projections. (A, B) Representative gating parameters of the FACS for identifying and isolating viable projecting neurons to the NAc in rats that received retro-tracer microinjections (A) or in controls (B). Pacific Blue-A represents the intensity of cell-viability dye, calcein violet; PE-A (phycoerythrin-area) represent the intensity of rhodamine dye contained in the retro-tracer. As such, Hi (high) population represent highly viable projection neurons, while Int (intermediate) are sub-viable or dying cells. (C) Representative RNA electrophoresis diagram showing high quality of RNA with a RNA integrity number (RIN) 8.4 (1-10 scale) extracted from a sorted PFC sample that contained 1376 events. 18s, 28s represent 18s, 28s ribosome RNAs. (D) Picture illustrates the PCR products on agarose gels (1.8%) after electrophoresis. cDNA were synthesized through RNA/cDNA amplification with mRNA extracted from sorted PFC or BLA neurons projecting to the NAc.

Experiment 7: Inhibiting endogenous PACAP signaling in the NAc increases cocaine-induced reinstatement. The above data raise the intriguing prospect that PACAP is a novel contributor to excitatory signaling in the NAc. To determine whether PACAP is an endogenous protective factor capable of limiting drug-seeking behavior, we examined whether a PACAP receptor inhibitor microinjected into the NAc would promote cocaine-seeking behavior. To do this, we modified the reinstatement paradigm to avoid potential ceiling effects of cocaine IP on reinstatement. Particularly, after the initial extinction training, rats received three cocaine reinstatement tests on separate days (Tests 1-3) so that behavior would approach extinction levels, thereby permitting sensitivity within the assay to detect increases in behavior. Next, PACAP6-38 was microinjected into the NAc and behavior was measured. PACAP6-38 microinjection in the NAc during Test 4 significantly increased cocaine-induced reinstatement compared to subjects receiving saline [Figure 4.9; $t(5) = 3.109$, $p < 0.05$), suggesting that endogenous PACAP in the NAc is an unrecognized factor influencing relapse vulnerability.

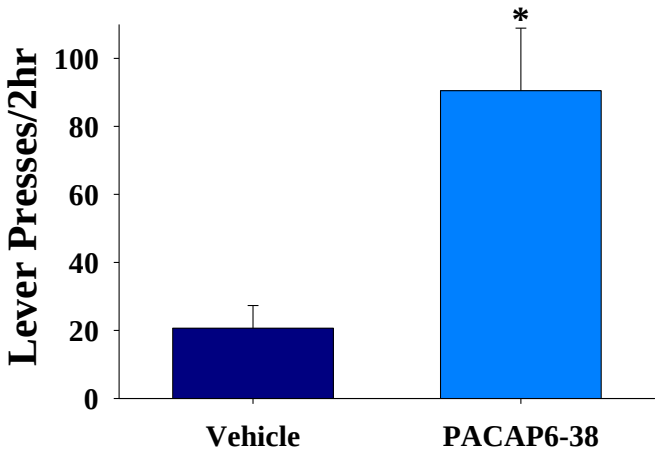

Figure 4.9. Inhibiting endogenous PACAP signaling in the NAc increases cocaine-induced reinstatement. Data depict lever responding during a two-hour session following intra-NAc microinjections of vehicle (saline) or PACAP6-38 (500 pmol) and a systemic injection of cocaine (10 mg/kg, i.p.) in rats (N=3-4/condition). * indicates a significant difference relative to vehicle group, T-test, $p < 0.05$.

Discussion

In this chapter, we found that PACAP depresses AMPAR-mediated EPSCs in the nucleus accumbens (NAc) medium spiny neurons (MSNs) projecting to the substantia nigra (SN). Moreover, this effect involves PACAP-induced upregulation of Sxc. This is the first finding that PACAP signaling regulates synaptic transmission in the NAc. The significance of this was evident by our finding that increasing PACAP signaling in the NAc significantly decreased cocaine-induced reinstatement of drug seeking. This effect of PACAP is abolished in Sxc loss-of-function mutants, suggesting the involvement of Sxc. In contrast, inhibiting PACAP receptors in the NAc exaggerated cocaine-seeking behavior, suggesting that endogenous PACAP in the NAc is a novel factor influencing relapse vulnerability. Next, we found that PACAP is expressed in NAc-projecting neurons in the prefrontal cortex (PFC) and basolateral amygdala (BLA). These are the first data demonstrating the existence of this peptide in corticostriatal projections to the NAc. Together, our data support the hypothesis that in the NAc, PACAP influences AMPAR-mediated synaptic transmission and plasticity through regulating Sxc. Such a function likely contributes to the coordinated glutamate network in the NAc and the proper expression of motivated behaviors.

The current study demonstrates that xCT and the primary PACAP receptor, PAC1R, are enriched in NAc astrocytes. These findings are consistent with a recent study conducted with mice cortical tissues (Zhang et al., 2014), suggesting that the enrichment of PAC1R and Sxc in *in vivo* astrocytes may be a general pattern in the CNS. The expression of PACAP signaling machinery in the NAc is consistent with the abundant immunoreactivity of this peptide in this brain region (Ghatei et al., 1993). To

investigate the cell-type specific expression of target genes, we used FACS with a newly developed transgenic rat model, which expresses membrane-targeted eGFP under GFAP control in order to better label astrocytes. The successful labeling of astrocytes is demonstrated by the lack of NeuN and abundant expression of GFAP mRNA in the GFP+ populations (Figure 4.1). Note that, even though the GFP- population is enriched in NeuN (e.g., relative to the total sample), it likely contains other cell populations, including astrocytes, microglia and oligodendrocytes. As these glia subtypes also express small amounts of Sxc (Zhang et al., 2014), this may explain the low level of xCT mRNA in GFP- samples.

Using two different approaches including FACS and combined immunohistochemistry and *in situ* hybridization, we found PACAP mRNA in the NAc afferents from the PFC and BLA. These findings support prior observations that PACAP is expressed primarily in glutamatergic neurons. In support, PACAP is co-expressed with vGluT2, and may be co-released with glutamate (Hannibal et al., 2000; Hannibal, 2002a; Engelund et al., 2010). This represents an important finding since neither this nor any other peptide has been implicated in the activity of NAc excitatory afferents. While it is unclear whether PACAP expression is exclusive to or ubiquitously expressed by all NAc excitatory afferents, our finding may have important implications for the cellular basis of cocaine-induced changes in the NAc. For example, cocaine-induced disruptions in glutamate homeostasis may be linked to cocaine-induced reductions in the activity of corticostriatal inputs to the NAc. This outcome may explain an interesting finding whereby manipulations in the cortex appear to be capable of normalizing astrocytic activity in the NAc (Berglind et al., 2009).

PACAP signaling in the NAc was found to depress AMPAR-mediated synaptic transmission. Interestingly, this function of PACAP appears to be circuit specific, as PACAP attenuates the AMPAR-EPSCs in SN-projecting MSNs, but not in VP-projecting MSNs. Moreover, this action of PACAP appears to be mediated by Sxc, since it is not observed in NAc tissues obtained from Sxc loss of function rats. This parallels prior observations in which astrocyte subpopulations in the striatum differentially regulate –SN and –VP projecting MSNs (Martin et al., 2015). Therefore, selective coupling to specific astrocyte subpopulations may add a layer of regulation for the MSNs which lead to the pathway-specific effects of PACAP.

Alternatively, efferent-specific gene expression may prime NAc-SN to undergo PACAP-induced regulation of synaptic transmission. In support, the MSNs involved in NAc-SN and NAc-VP pathways express heterogeneous biochemical profiles. For example, these pathways display differential expression of D1- and D2-like receptors (Lu et al., 1998; Gerfen and Surmeier, 2011). Interestingly, dopamine receptor activation may oppose PACAP-induced regulation of GluA1, which may provide a molecular basis for PACAP-induced signaling in one pathway but not the other. In addition, there may be other biochemical differences in neurons that account for this effect. For example, PAC1R is expressed in the SN-projecting neurons, while it is absent in the VP-projecting neurons (see Figure 5.2). In any of the above scenarios, the regulation of synaptic transmission in the NAc efferents likely results from the collective actions of PACAP on both astrocytes and neurons.

Consistent with others' findings that Sxc facilitates postsynaptic removal of AMPAR, resulting in the attenuation of synaptic strength (Williams and Featherstone,

2014), we found that application of PACAP to the NAc, which potentially increases Sxc activity, suppresses AMPAR-mediated synaptic transmission and reduces the phosphorylation of T840-GluA1, which is positively linked to AMPAR trafficking and channel conductance (Delgado et al., 2007; Jenkins et al., 2014). Given that the T840 site is a substrate for NMDAR-mediated signaling (Delgado et al., 2007; Lee et al., 2007; Gray et al., 2014), it is possible that glutamate from Sxc activates the extrasynaptic NMDARs which in turn reduce the phosphorylation of GluA1-T840. In support of these links, we found that PACAP-induced depression of AMPAR-EPSCs is abolished by Ro 25-6981, a selective antagonist for GluN2B-containing NMDARs, which are high-affinity glutamate receptors that can be tonically activated by astrocytes and expressed extrasynaptically (Barria and Malinow, 2002; Yoshii et al., 2003; van Zundert et al., 2004; Herman and Jahr, 2007; Le Meur et al., 2007; Hamilton and Attwell, 2010; Petralia et al., 2010; Gladding and Raymond, 2011; Paoletti, 2011; Povysheva and Johnson, 2012; Paoletti et al., 2013).

Interestingly, Ro 25-6981blocked PACAP-induced depression of AMPAR-EPSCs even though the MSNs were voltage-clamped at -80 mV, which is near their resting membrane potential. We used these parameters because it was found that astrocyte-induced regulation of GluN2B-NMDARs is voltage- and Mg^{2+}-independent (Hahn et al., 2015). Hence these data support the possibility that PACAP promotes astrocyte-neuron signaling (and/or signaling between extrasynaptic-synaptic microdomains) under resting conditions. This supports the possibility that signaling between astrocytes and neurons (or between extrasynaptic and synaptic domains) influences synaptic transmission even in cells or synapses that have not received recent synaptic inputs, which is important since

astrocytes have been implicated in regulating the firing of distal synapses or even entire networks of neurons (Fellin et al., 2004; Pascual et al., 2005; Fellin et al., 2006a). While beyond the scope of this study, this could be due to several possibilities. First, the voltage response in dendrites to the evoked synaptic inputs may have enabled the removal of the Mg^{2+} block of proximal NMDARs (Palmer and Stuart, 2009; Williams and Wozny, 2011; Acker et al., 2016). While this could contribute to our observations, it would not account for the observations of Hahn et al. (2015) since neurons in their study did not received synaptic (i.e., evoked) inputs. Second, astrocytes (and PACAP) may promote the activity of NMDARs that are incompletely blocked by Mg^{2+}. This could be due to fluctuations in local Mg^{2+} concentrations, which seems more likely in extrasynaptic microdomains since astrocytes are thought to regulate extracellular ion concentrations. Unfortunately, the biology of Mg^{2+} transporters is poorly understood (Ferre et al., 2011). It is shown that these transporters are enriched in astrocytes (Zhang et al., 2014), yet it is unclear if they are restricted to mitochondria in these cells. Alternatively, our finding could involve NMDARs that are partially resistant to Mg^{2+} blockade (e.g., triheteromeric GluN1-GluN2B-GluN2D-NMDARs) (Huang and Gibb, 2014). Lastly, this could indicate that the action of neuronal PACAP receptor signaling may include the removal of Mg^{2+} from NMDARs. In support of this compelling possibility, several studies have shown that PKC, which can be driven by PACAP receptors, is capable of removing Mg^{2+} blockade of NMDARs (Chen and Huang, 1992; Tyszkiewicz et al., 2004; Moriguchi et al., 2007). Regardless, these findings illustrate the potential complexity that exists in the striatum, and suggest that PACAP regulates synaptic transmission through upregulating Sxc activity and ensuring glutamate release from astrocytes, which activates extrasynaptic

NMDARs and subsequently inhibits AMPAR function. This multi-cellular mechanism may also underlie a number of findings in different brain regions where PACAP regulates phosphorylation of GluA1 and synaptic strength (Kondo et al., 1997; Roberto and Brunelli, 2000; Roberto et al., 2001; Ciranna and Cavallaro, 2003; Costa et al., 2009; Gardoni et al., 2012; Toda and Huganir, 2015). However, further studies are needed to validate these links.

Consistent with the behavioral outcome that would be predicted when suppressing the activity of NAc-SN MSNs (Rogers et al., 2008; Kravitz et al., 2012; Bossert et al., 2013; Macpherson et al., 2014), we found that PACAP microinjected into the NAc significantly attenuates cocaine-primed reinstatement. Consistent with our above findings, PACAP-induced regulation of behavior is likely mediated by Sxc since it is not evident in MSxc rats. Collectively, these data may establish PACAP signaling in the NAc as a critical protective factor capable of limiting drug-induced relapse. In support, we found that inhibiting endogenous PACAP signaling in the NAc following micro-injection of the PACAP receptor inhibitor PACAP6-38 augmented cocaine-induced reinstatement. Hence, these data indicate that heightened drug seeking may result from impaired PACAP signaling, which needs to be examined in future studies.

In conclusion, our data establish PACAP signaling in the NAc as a novel, key component of excitatory signaling cocaine seeking behavior. Moreover, PACAP is present in corticostriatal neurons, which are NAc afferents that are known to display persistent hypofunction following long-term cocaine administration (Jentsch and Taylor, 1999; Naqvi and Bechara, 2010; Goldstein and Volkow, 2011). These collective results may yield novel opportunities to understand the biological basis of addiction. In support,

human cocaine abusers exposed to craving-inducing stimuli exhibit increased activation of excitatory circuits originating in cortical regions, including orbital and prefrontal cortex, and projecting to the ventral striatum (Breiter et al., 1997; Volkow et al., 2005). Preclinical data also indicate the existence of drug-induced plasticity leading to activation of corticostriatal pathways. Activation of these circuits results in heightened extracellular glutamate in the NAc (Pierce et al., 1996; Reid and Berger, 1996) and stimulation of ionotropic glutamate receptors, both of which are necessary for cocaine primed reinstatement (Cornish and Kalivas, 2000; McFarland et al., 2003). Thus, these data raise the provocative possibility that PACAP signaling may be altered by cocaine. If so, this could have tremendous implications in understanding the pathological basis of cocaine addiction, since PACAP signaling regulates Sxc activity, NMDAR and AMPAR signaling, dendritic spine morphology (Gardoni et al., 2012), and LTP & LTD (Roberto and Brunelli, 2000) – all of which are altered in the NAc by cocaine.

Beyond cocaine addiction, our findings may have implications for understanding other CNS conditions given the role of glutamate as the primary excitatory signal in the brain. Moreover, our findings are consistent with the emerging viewpoint that normal excitatory signaling involves the highly integrated activity of multiple cell types in the brain, including astrocytes and neurons. These findings parallel compelling studies demonstrating that astrocyte-neuron communication may underlie the activity patterns of entire neural networks (Caudle, 2006; Volman et al., 2007; Pereira and Furlan, 2010; Amiri et al., 2013; Dallerac and Rouach, 2016). In fact, a recent report found that astrocytes contribute to gamma oscillations and recognition memory (Lee et al., 2014). It has been suggested that astrocytes may enable a high-degree of coordination over entire

populations of neurons due to several key morphological and structural features including the non-overlapping domains of these cells and the high number of synapses that can be contacted by an individual astrocytes (which may number in the millions in the human cortex) (Bushong et al., 2002; Halassa et al., 2007; Robertson, 2013, 2014). While astrocytes release a number of neuro-active substances that may underlie this important activity (Araque et al., 1999; Haydon, 2001; Perea and Araque, 2005; Fellin et al., 2006a; Haydon and Carmignoto, 2006; Araque et al., 2014; Perez-Alvarez et al., 2014), glutamate is clearly involved in this process. Hence, it is possible that the unique form of neuron-astrocyte signaling identified in these studies may contribute to the activity of entire neural networks, and changes in PACAP signaling may contribute to pathological states in a variety of CNS conditions.

CHAPTER V

DISCUSSION

General Discussion

Drug addiction is a chronic central nervous system (CNS) disorder characterized by persistent relapse susceptibility, even after years of abstinence (Jaffe et al., 1989; Withers et al., 1995). This results at least in part from drug-induced changes in glutamate signaling, especially within the corticostriatal projections to the nucleus accumbens (NAc) (Pierce et al., 1996; Reid and Berger, 1996; Breiter et al., 1997; Volkow et al., 1999; Cornish and Kalivas, 2000; Park et al., 2002; Baker et al., 2003; McFarland et al., 2003; McFarland et al., 2004; Kalivas and Volkow, 2005; Schmidt et al., 2005; Volkow et al., 2005; Madayag et al., 2007; Kau et al., 2008; Kalivas, 2009; Ary et al., 2013). While it is reasonable to predict that these discoveries could be leveraged to develop novel approaches to treat drug addiction, a challenge in revealing the molecular or cellular basis of pathological glutamate transmission in addiction (or any other CNS disorder) is that excitatory neurotransmission is comprised of a complex network of glutamate transporters, receptors, and release mechanisms expressed by multiple cell types, including neurons and astrocytes (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997; Danbolt, 2001; Schoepp, 2001; Baker et al., 2002; Baker et al., 2003; Pirttimaki et al., 2011; Bridges et al., 2012a). Moreover, while it is highly likely that glutamatergic signaling across distinct cell types is highly coordinated, little is known about the molecular basis of integrated astrocyte-neuron signaling.

These studies investigated the molecular basis of orchestrated neuron-astrocyte communication within the context of glutamate transmission in the NAc. To do this, I primarily focused on the regulation of an astrocytic glutamate release mechanism, system x_c- (Sxc) by neurons and a neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP). Sxc was chosen, since this non-canonical glutamate transporter is strongly linked to glutamate signaling in the NAc, and because cocaine-induced changes in Sxc are implicated in pathological changes in neuronal synaptic transmission (Baker et al., 2003; Madayag et al., 2007; Moussawi et al., 2009; Amen et al., 2011; Moussawi et al., 2011; Schmaal et al., 2012). As such, we were confident that glutamate released by Sxc contributes to astrocytic regulation of neuronal glutamate signaling. In turn, we examined the capacity for PACAP to sub-serve neuronal regulation of astrocytes, since this peptide is exclusively expressed by neurons in the CNS and has been demonstrated to regulate glutamate release and clearance by astrocytes (Figiel and Engele, 2000; Goursaud et al., 2008; Resch et al., 2014b; Zhang et al., 2014). Therefore, the intersection of PACAP and Sxc on the regulation of astrocytic and neuronal glutamate systems presents itself as a unique opportunity to understand the importance of neuron-astrocyte communication to synaptic transmission and behavior.

The studies in this dissertation demonstrate that glutamate release from astrocytes via Sxc is regulated by neuronal soluble factors (Chapter II), which likely involves the neuropeptide PACAP. In support, inhibiting PACAP signaling reversed the neuronal upregulation of Sxc activity in astrocytes, while PACAP application mimicked the actions of neuronal supplementation of astrocyte cultures. Interestingly, PACAP-induced regulation of Sxc was observed in both cortical and striatal astrocytes (Chapters II and

III), which supports the hypothesis that this unique form of neuron-astrocyte communication may be of relevance to excitatory signaling in each of these brain regions. Having found that PACAP is expressed in corticostriatal inputs to the NAc, we then sought to determine whether this neuropeptide is a novel component of excitatory signaling in this structure (Chapter IV). We found that PACAP application to NAc slices depressed synaptic transmission in neurons projecting to the substantia nigra (SN), an important efferent that is thought to encode positive reinforcement or motivated behaviors (Rogers et al., 2008; Kravitz et al., 2012; Bossert et al., 2013; Macpherson et al., 2014). Interestingly, PACAP-induced control over synaptic transmission required upregulation of Sxc activity. To demonstrate the *in vivo* relevance of each of these findings, we then found that microinfusion of PACAP into the NAc attenuated cocaine-primed reinstatement of extinguished drug seeking in rats (Chapter IV). Collectively, this dissertation reveals that a novel form of neuron-astrocyte communication, namely PACAP-induced regulation of Sxc, is a critical link integrating the glutamate network to permit coordinated excitatory transmission that underlies motivated behaviors and likely many other CNS functions.

Making the Human Brain Great Again: Critical Roles for Neurons and Astrocytes

In considering the importance of the above findings, it is interesting to examine our results through the contemporary perspective that the profound capabilities of the human brain stem from its extraordinary computational capacity as opposed to the size or other potentially unique features that have been historically used to explain why the human brain is so special (Roth and Dicke, 2005; Pereira and Furlan, 2010; Robertson,

2013, 2014; Dicke and Roth, 2016). In the study of the computational capacity of the brain (McCulloch and Pitts, 1990; Quian Quiroga and Panzeri, 2009), mathematical models demonstrate that even simplified neuron-neuron signaling that sum ***binary*** inputs to yield ***binary*** outputs is capable of performing logical tasks that are needed for information processing. This simplistic depiction does not fully account for synaptic integration, the computational process by which an individual neuron processes its synaptic inputs and converts them into an output signal. The reason for this is that the inputs and outputs of neurons are not necessarily binary. Towards this, the complexity of human neurons (e.g., extensive dendritic fields enabling important increases in the number of synaptic inputs per cell) has been suggested to account for the extraordinary computational capabilities of the human brain (Roth and Dicke, 2005; Dicke and Roth, 2016). In addition, local processing at axonal terminals (i.e., at the level of the presynaptic neuron) can also confer a degree of additional informational processing. Despite these additional forms of neuronal synaptic integration, it is doubtful that the profound capabilities of the human brain solely stem from neuron-neuron signaling.

In more recent efforts, attempts to model the extraordinary capabilities of the human brain have done so by incorporating astrocytes. By some estimates, astrocytes are the most abundant cell type in the brain (Barres, 2008). While classically depicted as metabolic support cells, work over the past few decades has definitively established astrocytes as key components of synaptic transmission and regulators of neural networks in the brain (Araque et al., 1999; Haydon, 2001; Perea and Araque, 2005; Fellin et al., 2006a; Haydon and Carmignoto, 2006; Araque et al., 2014; Perez-Alvarez et al., 2014). Within the context of computational capacity, it is important to note that astrocytes are

receptive to virtually every known neurotransmitter (NT), may contact millions of synapses, and are capable of releasing numerous neuro-active molecules, including glutamate (Bushong et al., 2002; Fellin et al., 2006a; Haydon and Carmignoto, 2006; Halassa et al., 2007; Verkhratsky, 2009; Robertson, 2013; Araque et al., 2014; Perez-Alvarez et al., 2014; Robertson, 2014).

Perhaps due to these attributes, mathematical models that incorporate neuronal-astrocyte communications portray an important role for astrocytes as reservoirs or storage banks of information flowing through neuronal networks (Caudle, 2006; Volman et al., 2007; Amiri et al., 2013; Dallerac and Rouach, 2016). This and other properties may be due to unique astrocyte features such as non-overlapping spatial domains and relatively slow intra-cellular signaling (at least as compared to neurons) (Bushong et al., 2002; Halassa et al., 2007; Robertson, 2013, 2014). Thus, at a minimum, the incorporation of astrocytes enables the expansion of temporal and spatial limitations of signal computation or integration (Caudle, 2006; Volman et al., 2007; Pereira and Furlan, 2010; Amiri et al., 2013; Dallerac and Rouach, 2016). Therefore, the inclusion of these cells is required to maximize the computational capacity of the human (or any other) brain. In other words, the study of these cells within the context of neuron-astrocyte and astrocyte-neuron communications (Figure 5.1A) will be essential for understanding the local information processing that underlies the output of a given brain region or related circuits.

The above concepts are useful in considering behavioral control by the NAc. To the extent that optimized patterns of behavior promote survival through interactions with the environment in a manner that increases biological fitness, then behavioral control by the NAc should require the integration of a great number of factors. Indeed, the NAc

receives diverse input signals originating from many cortical areas, as well as from the hippocampus, amygdala, midbrain dopamine nuclei and hypothalamus (Salgado and Kaplitt, 2015). Each of these inputs encodes key behavioral antecedents, such as decision making (executive function), memory, emotion, environmental salience, internal states and more. As such, our findings may be framed as demonstrating that presynaptic neurons recruit astrocytes into the local integration of incoming information through the release of PACAP (Figure 5.1B). Moreover, astrocytes then can shape the output from the NAc in part through the release of glutamate from Sxc. Hence, our work may have identified an important loop of communication between neurons and astrocytes in a manner that promotes the integrative capacity of local signaling in the NAc for the purpose of behavioral control. These key points will be discussed within a functional unit

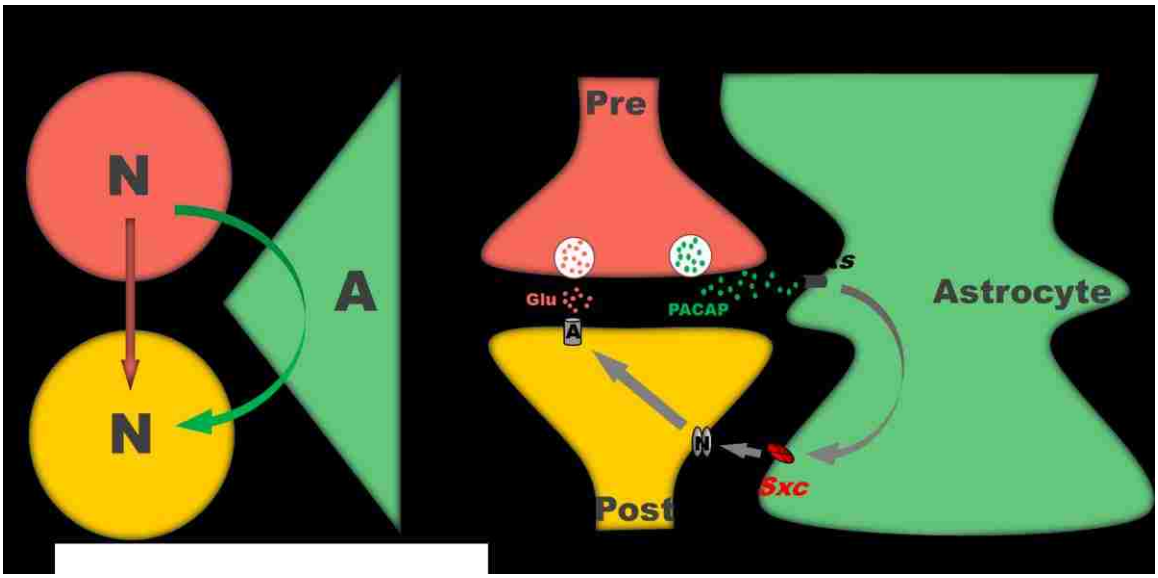

Figure 5.1. A typical neurotransmission unit. (A) Conceptual illustration of an information processing unit that incorporates an astrocyte. N: neuron; A: astrocyte. (B) Illustration of the major findings of this dissertation in a tripartite synapse in the NAc. Pre: presynaptic terminal; Post: postsynaptic terminal; Glu: glutamate; PRs: PACAP receptors; Sxc: system x_c-.

of neurotransmission/information processing, namely, a tripartite synapse in the NAc that is formed by a presynaptic neuron from the prefrontal cortex (PFC), a postsynaptic

medium spiny neuron (MSN) projecting to the SN, and the adjacent astrocyte process (Figure 5.1B). As such, the remaining discussion is organized by factors related to a) presynaptic neuronal regulation or signaling to astrocytes and b) astrocytic regulation or signaling to postsynaptic neurons.

Presynaptic Neuronal Recruitment of Astrocytes

In these studies, we observed the expression of PACAP mRNA in cortical neurons both *in vitro* (Chapter II) and *in vivo* (Chapter IV), and this represents the first demonstration that corticostriatal projections to the NAc contain PACAP (Chapter IV). In addition, we observed PACAP mRNA in glutamatergic projections to the NAc from the basolateral amygdala. Lastly, we detected extracellular PACAP in the NAc using peptide microdialysis (data not shown). Collectively, these findings are consistent with other reports suggesting that PACAP is often, if not exclusively, expressed in glutamatergic neurons and is released as a neurotransmitter through the same nerve terminals as glutamate (Koves et al., 1994; Hannibal et al., 2000; Hannibal, 2002b, a; Stumm et al., 2007; Engelund et al., 2010; Hu et al., 2011;Masuo et al., 1992; Masuo et al., 1993; Golombek and Rosenstein, 2010; Purrier et al., 2014).

Co-transmission increases computational capabilities of the neural network

The release of PACAP and glutamate from the same neurons represents a phenomenon that is increasingly recognized to occur throughout the CNS, and this diversifies the manner in which inputs can alter activity within target regions or circuits (Seal and Edwards, 2006; Broussard, 2012; Hnasko and Edwards, 2012). On one hand,

this diversification can involve spatial parameters. For example, the cellular targets of glutamate and PACAP may be at least partially distinct. It is widely thought that the primary target of synaptically released glutamate is the postsynaptic neuron and that there are numerous mechanisms in place to limit spillover (Logan and Snyder, 1971; Balcar and Johnston, 1972; Danbolt, 2001; Bridges et al., 2012a). Hence, glutamate may be especially important for neuron-neuron signaling. Alternatively, the primary PACAP receptor, PAC1R is mainly expressed by astrocytes (Chapter IV). While we have observed its presence in SN-projecting MSNs (Figure 5.2), I found that the total

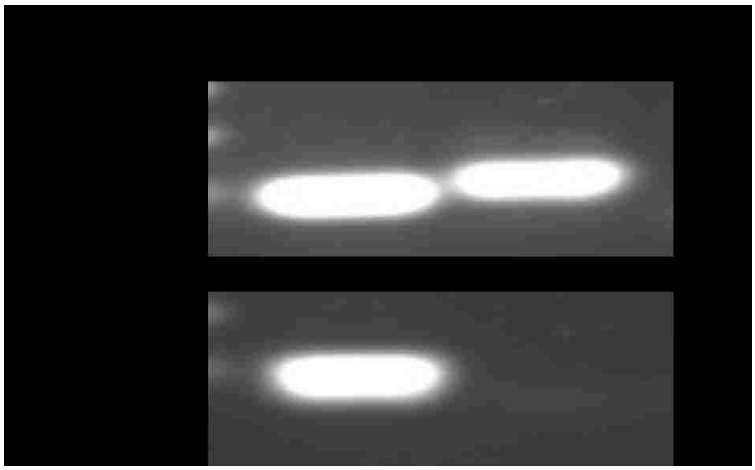

Figure 5.2. PAC1R is expressed in NAc neurons projecting to the substantia nigra, but not to the ventral pallidum. Picture illustrates PCR products on an agarose gel (1.8%) after electrophoresis. cDNA was synthesized through RNA amplification and reverse transcription with mRNA extracted from circuit-specific neurons in the NAc that were obtained via fluorescence-activated cell sorting (FACS). PCR was conducted with cDNA templates and primers specific to rat GAPDH and PAC1R. Presence of bands at expected sizes indicates the expression of the target genes. SN: substantia nigra; VP: ventral pallidum.

proportion of PAC1R present in astrocytes from NAc tissues exceeds 95% (Chapter IV). This is a level of cell-specific expression that is rarely observed for a neurotransmitter receptor, especially in adult, fully-developed cells. Therefore, glutamate and PACAP released from corticostriatal inputs to the NAc may differentially target postsynaptic and astrocytic signaling, with glutamate favoring the postsynaptic terminal, while PACAP

favoring the astrocyte process. In support, a loss of Sxc function completely eliminated the capacity of this neuropeptide to regulate glutamate release from striatal astrocytes, synaptic transmission in the NAc and cocaine reinstatement (Chapter IV). In addition, PACAP may also increase the temporal aspects of information encoded by incoming corticostriatal (or other) inputs, given that neuropeptide transmission often permits increased temporal domains (Nusbaum et al., 2001; van den Pol, 2012). As such, the co-transmission of transmitters (e.g. glutamate and PACAP) provides strong support for the idea that this novel form of neuron-astrocyte signaling has the benefit of enhancing the information processing capabilities within local NAc circuits.

Co-transmission in the motive circuit

Interestingly, co-transmission has been demonstrated in other parts of the motive circuit. For example, it is shown that VTA (ventral tegmental area) dopaminergic afferents to the striatum co-release (i.e., transmitted through the same vesicles) GABA (Tritsch et al., 2012). Similarly, it has been demonstrated that VTA neurons projecting to the NAc express both tyrosine hydroxylase and vesicular glutamate transporter 2 (vGluT2), suggesting the co-transmission of dopamine and glutamate (Chuhma et al., 2009; Stuber et al., 2010; Tecuapetla et al., 2010). Alternatively, substance P and enkephalin that are preferably expressed in, respectively, D1- and D2-like receptor-expressing MSNs in the striatum (Lu et al., 1998; Steiner and Gerfen, 1998) co-transmit with GABA, and have been shown in modulating GABAergic transmission (Maneuf et al., 1994; Tan and Bullock, 2008; Govindaiah et al., 2010; Tritsch et al., 2016). In contrast to these, our findings may be the first implicating the co-transmission of a

neuropeptide with the primary excitatory neurotransmitter glutamate in corticostriatal (and other excitatory) projections of the motive circuit.

PACAP receptors: the gate to astrocyte information storage.

In these studies, PACAP signaling in astrocytes is considered to be an important form of astrocytic regulation of incoming information. As such, we sought to identify the precise PACAP receptor(s) involved in this phenomenon. PACAP binds to three PACAP receptors (PAC1R, VPAC1R and VPAC2R) with similarly high affinities (Harmar et al., 1998; Harmar, 2001; Vaudry et al., 2009). In an initial attempt to identify the relevant receptor(s), we first examined which receptors are expressed by cortical and striatal astrocytes. Having detected all three PACAP receptors in cortical and striatal astrocytes (Chapters II and III), a finding that is consistent with others (Ashur-Fabian et al., 1997; Magistretti et al., 1998; Grimaldi and Cavallaro, 1999; Joo et al., 2004; Masmoudi-Kouki et al., 2007; Nishimoto et al., 2007; Nishimoto et al., 2011; Zhang et al., 2014), we then examined expression patterns in adult NAc astrocytes. We found that both PAC1R (Chapter IV) and VPAC2R are present in NAc astrocytes (Figure 5.3), indicating that the

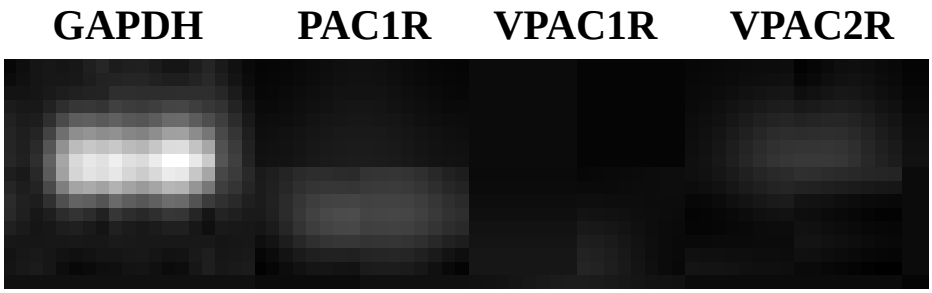

Figure 5.3. PAC1R and VPAC2R are expressed in adult NAc astrocytes. Picture illustrates PCR products on an agarose gel (1.8%) after electrophoresis. cDNA was synthesized through reverse transcription with mRNA extracted from adult NAc astrocytes obtained via FACS. PCR was conducted with cDNA templates and primers specific to rat PAC1R, VPAC1R and VPAC2R. Presence of bands at expected sizes indicates the expression of the target genes.

expression of PAC1R and VPAC2R persists beyond any potential developmental changes. While VPAC1R appears to be undetectable in NAc astrocytes (Figure 5.3), it may be a false negative result caused by its low abundance, especially in astrocytes, and the limitation of the detecting method (i.e. PCR). In support, it has been shown that PAC1R transcripts are much more abundant than either VPAC1R or VPAC2R, especially in astrocytes, with the respective FPKM (fragments per kilobase of transcript per million mapped reads) values to be > 150, < 0.2 and ≈ 2 (Zhang et al., 2014). Moreover, both PAC1R and VPAC2R appear to be more enriched in astrocytes than in neurons, with ratios at 10:1 (PAC1R) and 5:1 (VPAC2R), while VPAC1R is more enriched in neurons, with a 5:1 ratio compared to astrocytes (Zhang et al., 2014). Because of this and related observations, we hypothesized that PAC1R may be the primary mediator for PACAP-induced regulation of astrocyte Sxc. In support, brief application of the PAC1R inhibitor PACAP6-38 reversed the capacity for neurons to promote Sxc activity in astrocytes (Chapter II).

However, we are confident that activation of VPAC receptors (VPACRs) is also sufficient to upregulate astrocytic Sxc. In support, we observed that vasoactive intestinal peptide (VIP), which activates both VPAC1R and VPAC2R (Dickson and Finlayson, 2009), significantly upregulates Sxc activity in cortical astrocytes (Figure 5.4A), suggesting that VPACRs are potentially involved, consistent with others' finding (Resch et al., 2014b).

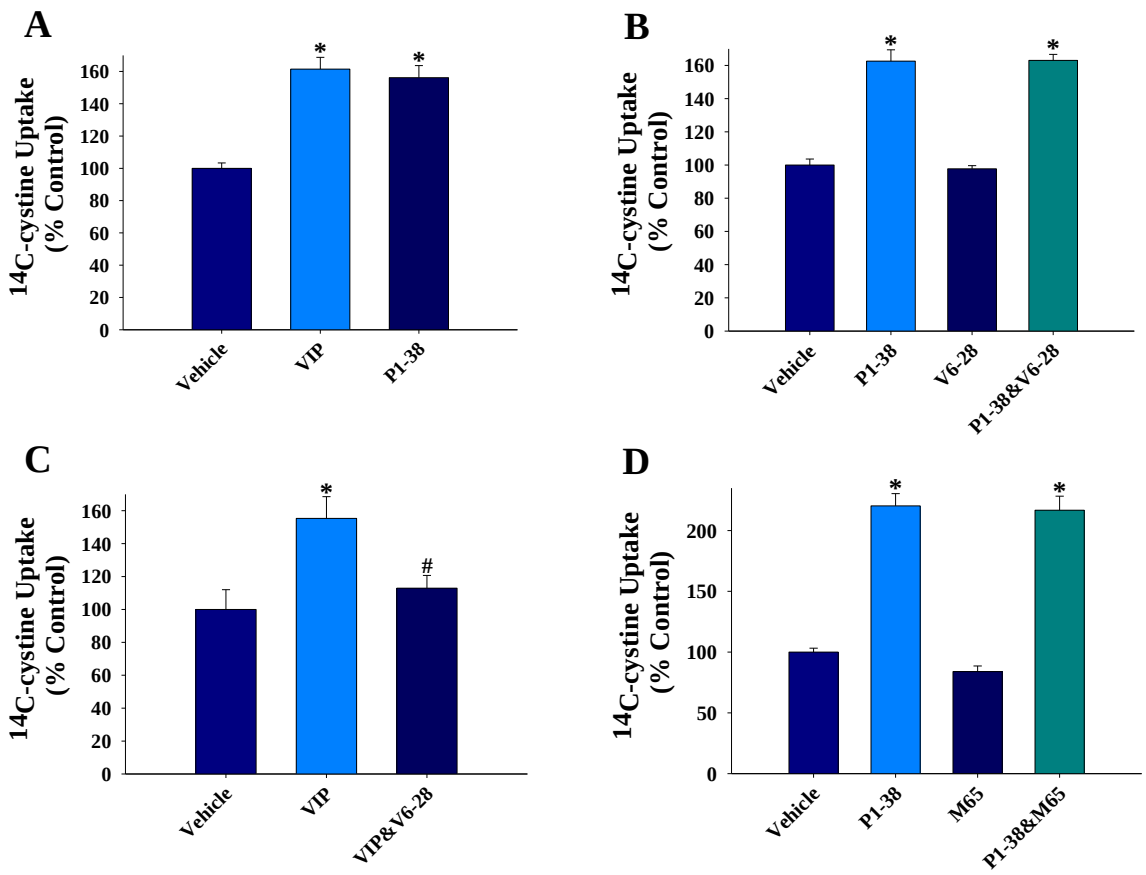

Figure 5.4. PACAP-induced regulation of Sxc activity is likely mediated by PAC1R and VPAC receptors. Data depict mean ± SEM levels of ^{14}C-cystine in purified rat cortical (A, B, C) or striatal (D) cultures after 24 hours treatment with vehicle, or ligands including VIP (10 nM), PACAP1-38 (designated as P1-38, 10 nM), VIP6-28 (designated as V6-28, 1 μM; VPAC1R and VPAC2R non-selective antagonist) and M65 (1 μM; PAC1R antagonist), or ligand mixtures including PACAP1-38 10 nM & VIP6-28 1 μM (designated as P1-38&V6-28), VIP 10 nM & VIP6-28 1 μM (designated as VIP&V6-28) and PACAP1-38 10 nM & VIP6-28 1 μM (designated as P1-38&V6-28) (N=6-12/condition). (A-D) * indicates a significant difference relative to vehicle-treated purified astrocyte cultures, Tukey HSD, $p < 0.01$. (C) # indicates a significant difference relative to VIP-treated purified astrocyte cultures, Tukey HSD, $p < 0.05$.

Surprisingly, a non-selective VPACR antagonist VIP6-28 failed to attenuate the augment of Sxc activity induced by PACAP (Figure 5.4B). One interpretation is that VIP6-28 lost its potency in blocking VPACRs. This possibility is unlikely, as we found that VIP6-28 significantly decreased VIP-induced upregulation of Sxc activity (Figure 5.4C). Alternatively, it is possible that both PAC1R and VPACRs are capable of

mediating PACAP-induced regulation of Sxc in cultured astrocytes, and their functions in this regard are redundant. Consistent with this idea, application of selective PAC1R antagonist M65 to striatal astrocytes was not able to attenuate the impact of PACAP.

Although PAC1R and VPACRs all appear to be sufficient in mediating PACAP-induced regulation of Sxc in cultured astrocytes, whether or not they are accountable for *in vivo* actions of endogenous PACAP is not clear. This is because that PACAP applied to cultured cells is likely capable of reaching to all different receptors through diffusion in the media; while *in vivo* PACAP may be only released through the presynaptic terminals, diffuse within a confined compartment and target astrocyte processes that express a defined population of PACAP receptors, e.g. PAC1R. Alternatively, PAC1R may be the only receptors expressed in the astrocyte domains facing PACAP releasing sites of corticostriatal terminals, while VPACRs are compartmentalized to be only activated by VIP from other inputs.

VIP-induced regulation of Sxc and its potential role in GABA transmission in the NAc

The above discussion supports an idea that VIP may be another critical endogenous regulator of Sxc, through which this peptide regulates synaptic transmission in the NAc. This is because, in addition to its capability of increasing Sxc activity in astrocytes (Figure 5.4A), VIP is highly expressed in the terminal field within the NAc (Sims et al., 1980). Moreover, we observed the expression of both VPAC1R and VPAC2R in cultured striatal astrocytes (Chapter III); VPAC2R has also been detected in adult NAc astrocytes (Figure 5.2).

In contrast to PACAP, which is located in glutamatergic neurons and is involved in regulating glutamate transmission (see the above discussion), VIP has been repeatedly linked to GABAergic neurons and shown to modify GABA signaling. For example, VIP is co-expressed with GABA in a major population of the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore et al., 2002). Similarly, VIP in the hippocampus is expressed exclusively by GABAergic interneurons (Acsady et al., 1996), and influences GABA release. Moreover, VIP concerts pre and postsynaptic GABAergic transmission in the hippocampus, which leads to the disinhibition of pyramidal cell dendrites and causes an enhancement of synaptic transmission (Cunha-Reis et al., 2004). These observations implicate that VIP signaling in the NAc may regulate GABA transmission, possibly through a similar mechanism to PACAP, namely, by inducing astrocyte Sxc. This could be an interesting and important direction for future studies.

However, the origin of the endogenous VIP in the NAc still needs to be determined. While some studies implicate that the majority of VIP in the NAc originates from its projections that are embedded in the medial forebrain bundle (Marley et al., 1981), others show that VIP is expressed in aspiny interneurons in rat striatum (Theriault and Landis, 1987). In contrast, I have observed abundant VIP mRNA in mixed striatal cultures containing neurons and astrocytes, and a lack of VIP in striatal astrocyte cultures (Figure 5.5). Therefore, similar to PACAP, VIP also appears to be expressed exclusively by neurons, consistent with others' finding (Zhang et al., 2014). In a similar manner to our results suggesting that neuronal PACAP increases xCT expression (Chapter II), neuron-derived VIP may contribute to the higher level of xCT mRNA that was observed in the

mixed striatal cultures compared to striatal astrocytes (Figure 5.6).Yet, whether VIP in striatal cultures originates from the interneurons or GABAergic MSNs is uncertain.

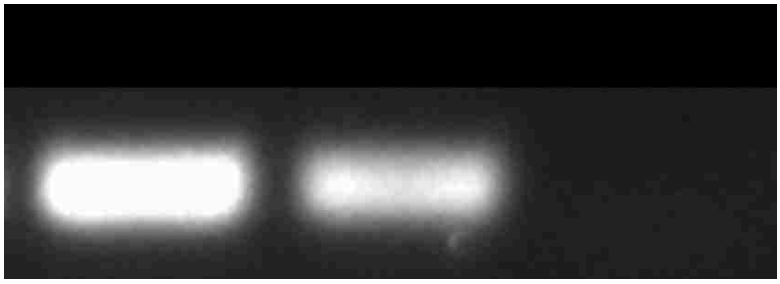

Figure 5.5. VIP is expressed in cultured striatal neurons but not astrocytes. Picture illustrates PCR products on an agarose gel (1.8%) after electrophoresis. cDNA was synthesized through reverse transcription with mRNA extracted from striatal cell cultures: STM, mixed striatal cultures; STN, striatal neurons; STA, striatal astrocytes. PCR was conducted with cDNA templates and a primer set specific to rat VIP. Presence of bands at the expected size indicates the expression of the target gene.

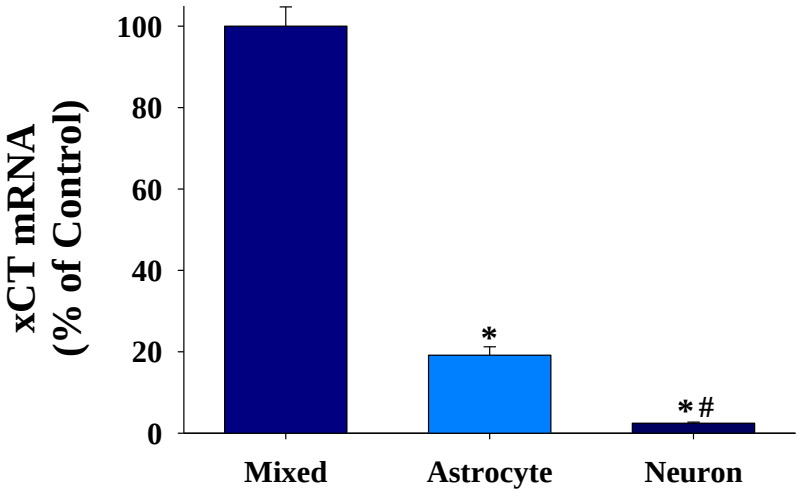

Figure 5.6. Striatal neurons increase xCT expression in striatal astrocytes. Data depict mean ± SEM levels of xCT mRNA in rat striatal cell cultures (N= 4/cell type). * indicates a significant difference relative to mixed cultures, Tukey HSD, $p < 0.001$; # indicates a significant difference relative to astrocyte cultures, Tukey HSD, $p < 0.01$.

Regardless of the uncertainty of the endogenous origin, the abundance of VIP signaling in the NAc appears consistent across different studies (Sims et al., 1980; Marley et al., 1981). These evidences discussed above and our observations implicate that VIP may regulate transmission in the NAc through its actions on astrocyte functions (e.g. Sxc).

The nature of PACAP-induced regulation of Sxc

In order to understand the potential contributions of phasic PACAP release to signal integration in the NAc, we became interested in the nature of PACAP-induced regulation of Sxc. Specifically, we were interested in learning whether brief PACAP application would be capable of rapidly (i.e., on a scale of minutes, see text box)

Time is often used a key variable in predicting whether changes in protein function reflect post-translational modification or changes involving gene expression. Thus, when altered protein function is observed in a manner of minutes, such as 6-min application of PACAP altering Sxc activity, we often exclude the possibility that changes in gene expression are involved. Is this a safe assumption? To examine this, we obtained published estimates of the key phenomena involved in protein expression.

Transcription - in eukaryotes, transcription has been reported to occur at a rate of 50-100 nucleotides per second (nt/s) (Dundr et al., 2002; Darzacq et al., 2007; Singh and Padgett, 2009), but this may be interspersed with pauses, which leads to a much slower rate at 6 nt/s (Darzacq et al., 2007).

Splicing of mRNA - the estimates of splicing are less detailed, but studies provide a timeframe of 5-10 minutes to excise introns from transcripts (Singh and Padgett, 2009).

Translation in eukaryotes requires an estimate of one second per 6 amino acids (Ingolia et al., 2011).

When applying the above estimates to the gene SLC7A11 that encodes xCT, it is apparent that it would take at least 75 seconds for transcription (74706 bp) (Gibbs et al., 2004), 5 minutes for splicing, and 85 sec for the translation (502 amino acids) (Alhaddad et al., 2014). This totals to 7-8 minutes, but it does not include the time needed for protein folding, trafficking, and insertion. Hence, it is unlikely that transcription is involved in PACAP-induced regulation of Sxc when applied for 30 min or less, especially since time is required to enable exogenous PACAP to diffuse to its receptor and alter intracellular physiology needed to regulate Sxc.

upregulating Sxc. We found that PACAP applied for as few as 6-30 min (Chapters III and IV) produced a significant increase in Sxc activity. This timescale likely reflects post-translational modification of xCT. However, the regulation of Sxc through post-translational modification is still poorly understood. While the xCT protein has been reported to contain consensus phosphorylation sites for various kinases (Baker et al., 2002), there has been only a single study clearly linking Sxc activity with phosphorylation (McClatchy et al., 2016). In this study, the mutagenesis of S26 on xCT, which corresponds to a phosphorylation sequence for mitogen and stress activated protein kinase (MKS), resulted in impaired Sxc activity. Our data are consistent with this observation in that MSK and its upstream activators PKA, ERK, and Epac are required for PACAP-induced regulation of Sxc (Chapter III). This is important, in part, since these signaling pathways have been implicated in cocaine addiction (Nestler, 2004; Kalivas and O'Brien, 2008; Besnard et al., 2011; Cahill et al., 2014; Liu et al., 2016), although these changes are often presumed to occur in neurons.

In addition to the rapid regulation, the magnitude of PACAP-induced increases in Sxc was significantly greater following long-term application, which also leads to increased xCT mRNA (Chapters II and III). This is of potential interest, given prior suggestions that astrocytes enrich local synaptic integration, in part by increasing the temporal dimension or persistence of incoming signals. Hence, it is possible that PACAP-induced Sxc regulation may occur along multiple timescales that reflect this important contribution of neuron-astrocyte communication. In support, we detected basal (i.e., unstimulated) PACAP levels in NAc microdialysis samples at a concentration of at least 83-267 pM. Note, these values were not corrected by our probe recovery rates,

which are likely less than 5%. Thus, basal PACAP levels may exist at a concentration as high as 5 nM. This is interesting, since it is in range of the nanomolar affinity of PACAP for PACAP receptors (Cauvin et al., 1990; Gottschall et al., 1990; Lam et al., 1990; Gottschall et al., 1991; Suda et al., 1992). Further, we observed PACAP-induced regulation of Sxc in striatal astrocytes at concentrations as low as 1 nM (Chapter III). Both PKA and Epac are also likely involved in PACAP-induced mRNA upregulation of xCT, given that they are capable of activating transcriptional factors (Mayr and Montminy, 2001; Schmidt et al., 2013). In addition, inhibition of PKA or Epac significantly attenuated 24-hr PACAP-induced increase of Sxc activity (Chapter III). However, more work is needed to understand the direct impact of PKA and Epac on xCT expression, and to what extent the increased expression of xCT versus post-translational modification contribute to increased Sxc activity observed following long-term PACAP treatment (e.g. 24 and 72 hr).

An important assumption in our interpretations up to this point is that Sxc activity *in vivo* is primarily if not exclusively astrocytic. This has been widely reported in the literature by measuring Sxc activity and/or cellular expression of xCT (Pow, 2001; Zhang et al., 2014). However, other authors suggest that neuronal cell lines and cultures may also display Sxc (Lewerenz et al., 2012a). To examine this, we determined the percentage of xCT mRNA obtained from adult rats that was contained in astrocytes versus every other type of cells. We observed that at least 90% of the total amount of xCT mRNA was present in our GFP+ samples following fluorescence-activated cell sorting (Chapter IV). Note, the remaining 10% reflects potential xCT mRNA expression in astrocytes not sorted into the GFP+ fraction (GFAP is detected in the GFP- fraction

since it is impossible to sort every astrocyte while excluding neurons in the GFP+ fraction), other glial cells, epithelial cells, and neurons. Importantly, we found that PACAP did not alter cystine uptake in neuronal cultures (Chapter II). As a result, our data strongly implicate astrocytic Sxc in our studies. Thus, we are confident that PACAP-induced regulation of Sxc in the NAc reflects neuron-astrocyte signaling. To determine whether these findings demonstrate that PACAP-induced regulation of Sxc in astrocytes is relevant to local synaptic integration, it then became necessary to demonstrate that PACAP-induced regulation of Sxc alters the output from the NAc and behavior.

Astrocyte-Neuron Regulation

In Chapter IV, we showed that PACAP depresses AMPAR-mediated EPSCs in NAc MSNs projecting to the SN. This is the first demonstration that PACAP signaling regulates synaptic transmission in the NAc, while similar effects have been shown in other brain regions, such as hippocampus and the suprachiasmatic nucleus (SCN) (Kondo et al., 1997; Roberto and Brunelli, 2000; Kopp et al., 2001; Roberto et al., 2001; Ciranna and Cavallaro, 2003; Costa et al., 2009; Gardoni et al., 2012; Toda and Huganir, 2015). Therefore, there was a need to establish the cellular basis for PACAP-induced regulation of synaptic transmission. While PACAP-induced regulation of synaptic transmission is often presumed to involve neuronal PACAP signaling, our data provide strong support for the involvement of astrocytes. In support, PACAP-induced regulation of AMPAR-mediated synaptic transmission in the NAc is not present in Sxc loss-of-function mutants, nor is the impact of PACAP on cocaine-induced reinstatement. Interestingly, our group

has begun collecting data demonstrating that re-introducing xCT cDNA into the NAc of these mutant rats rescues PACAP-induced blockade of cocaine reinstatement. Moreover, we and others have found that the elimination of Sxc in rats and/or mice produces deficits at the molecular, cellular, and behavioral levels that are opposite to what we have observed with PACAP and have attributed to increased Sxc. This includes the attenuation of synaptic strength involving AMPARs, reduced levels of basal glutamate, heightened phosphorylation of GluA1-T840, and augmented cocaine reinstatement (Baker data not shown; Williams and Featherstone, 2014; De Bundel et al., 2011; Li et al., 2012).

Encoding astrocyte-neuron signaling: A possible role for extrasynaptic NMDARs

Having established that PACAP-induced increases in Sxc contribute to the actions of PACAP in the NAc, we next examined the glutamate receptor that may encode this unique form of neuron-astrocyte signaling. Outside the NAc, PACAP-induced regulation of AMPAR signaling appears to be NMDAR-dependent. In support, potentiation of NMDAR-mediated synaptic activity by PACAP has been repeatedly demonstrated (Liu and Madsen, 1997; Wu and Dun, 1997; Harrington et al., 1999). This effect likely involves GluN2B-containing NMDARs. For example, PACAP promotes the phosphorylation of GluN2B, and in doing so enhances NMDAR-mediated currents, (Yaka et al., 2003; Macdonald et al., 2005; Resch et al., 2014a). Moreover, PACAP-induced dephosphorylation of GluA1-T840 in the hippocampus is mediated by NMDARs (Toda and Huganir, 2015). This effect is consistent with several studies implicating that NMDARs promote the dephosphorylation of GluA1-T840 of AMPARs, possibly through induction of protein phosphatase 1- and/or 2A (Delgado et al., 2007; Lee et al., 2007;

Gray et al., 2014). Similar to work in the hippocampus (Toda and Huganir, 2015), we also found that PACAP-induced regulation of synaptic transmission in the NAc requires activation of GluN2B-containing NMDARs, as co-application of a GluN2B-selective NMDAR antagonist Ro 25-6981 blocked PACAP-induced depression of AMPAR-mediated EPSCs (Chapter IV).

Given the above findings, we are confident that PACAP-induced regulation of astrocytic Sxc regulates synaptic transmission in NAc efferents by supplying glutamate capable of activating neuronal GluN2B-containing NMDARs. Moreover, we believe that the NMDARs encoding PACAP signaling through astrocytes are located extrasynaptically (Figure 5.1B). First, extrasynaptic NMDARs are often thought or have been shown to be GluN2B containing (Papouin et al., 2012). In support, GluN2B preferably associates with scaffolding proteins SAP102 (Yoshii et al., 2003; van Zundert et al., 2004), which is primarily located outside of synaptic domains (Barria and Malinow, 2002; van Zundert et al., 2004; Petralia et al., 2010; Gladding and Raymond, 2011). Further, extrasynaptic but not synaptic NMDARs are thought to inhibit AMPAR-mediated EPSCs (Papouin and Oliet, 2014). Lastly, several studies have implicated extrasynaptic but not synaptic NMDARs in tonic regulation by either non-vesicular and/or astrocytic glutamate release (Jabaudon et al., 1999; Warr et al., 1999; Angulo et al., 2004; Fellin et al., 2004; Shigetomi et al., 2008; Bardoni et al., 2010; Nie et al., 2010). Consistent with this, extrasynaptic but not synaptic NMDARs are often found to be clustered at sites proximal to astrocytic processes (Petralia et al., 2010). Given that non-vesicular release from Sxc has been implicated as the major source of extrasynaptic glutamate in brain regions including the NAc (Baker et al., 2002; De Bundel et al., 2011;

Massie et al., 2011), it is possible that this form of neuron-astrocyte-neuron signaling may be present in the NAc and other structures.

A possible role in astrocyte-mediated neuronal synchronization

We speculate that the PACAP-induced regulation of Sxc and extrasynaptic NMDARs may not be limited to the synapse where PACAP is released, but underlie the expanded spatial and temporal influence of an astrocyte on multiple local neurons, for example, in inducing neuronal synchronization. This is because that glutamate released from astrocytes has been shown to activate extrasynaptic NMDARs on the postsynaptic terminals and induce characteristic slow inward currents (SICs) that are synchronized in local neurons (Parri et al., 2001; Angulo et al., 2004; Fellin et al., 2004; Fellin et al., 2006b; D'Ascenzo et al., 2007). Notably, in these studies, glutamate release results from spontaneous or evoked calcium oscillations in astrocytes. Yet, whether or not a calcium-independent astrocyte glutamate release mechanism, such as Sxc, is capable of inducing SICs has not been investigated. Moreover, it is shown that SICs and neuronal synchronization could be induced by stimulating the afferents (Pirttimaki et al., 2011), suggesting that the astrocyte glutamate release mechanism that is involved in SIC generation and synchronization is regulated by neuronal activity. Along with the above discussion, these evidences implicate that neuronal PACAP-induced glutamate release through astrocyte Sxc and subsequent extrasynaptic NMDAR activation may also be capable of influencing local neuronal network (e.g. synchronizing a number of local neurons). This could be achieved by different mechanisms, for example, PACAP-induced signaling cascades from one astrocyte process spread within the astrocyte and regulate Sxc on other processes that encapsulate neighboring synapses and neurons. Alternatively,

the increased spatial domains of neuropeptide transmission may allow PACAP to target Sxc on multiple astrocyte terminals and thus to influence multiple tripartite synapses simultaneously.

Interestingly, synchronization of SICs induced by stimulating afferents could sustain an extended time, for example, from minutes to one hour after the initial stimulus (D'Ascenzo et al., 2007; Pirttimaki et al., 2011). Given the expanded temporal domain of neuropeptide mediated regulation, it is possible that phasic PACAP release in response to neuronal activity is capable of inducing sustained Sxc upregulation, which contributes to the prolonged impact of astrocytes on neuronal SICs. Yet, these ideas are highly speculative and further investigation is needed to determine whether rapid effect of PACAP on Sxc activity is long-lasting.

Impaired Neuron-Astrocyte-Neuron Communication: Implications for CNS Pathology

Drug addiction is a complex, multi-faceted disorder characterized by compulsive drug seeking even when such behavior results in profound negative consequences to the individual. The chronic aspect of the disease involves episodes of heightened relapse vulnerability even following long periods of abstinence, which stem from persistent cocaine-induced neuroadaptations that disrupt glutamate transmission (Jaffe et al., 1989; Withers et al., 1995; Baker et al., 2003; Kalivas and Volkow, 2005; Nestler, 2005; Hyman et al., 2006; Koob and Volkow, 2010). Of particular importance, hypofrontality (e.g., reduced basal activity) in the PFC and other NAc-related maladaptation have been linked to behavior in virtually every preclinical model of drug seeking and has been found to correlate with the severity of drug craving in human substance abusers (Pierce et

al., 1996; Breiter et al., 1997; Jentsch and Taylor, 1999; Volkow et al., 1999; Park et al., 2002; Baker et al., 2003; McFarland et al., 2003; McFarland et al., 2004; Schmidt et al., 2005; Madayag et al., 2007; Kau et al., 2008; Naqvi and Bechara, 2010; Goldstein and Volkow, 2011; Ary et al., 2013).

To the extent that corticostriatal inputs are an important source of NAc PACAP, then cocaine-induced hypofrontality may indicate the existence of cocaine-induced disruptions in PACAP signaling. Interestingly, hypofrontality is also observed in rats using the cocaine reinstatement model (Chen et al., 2013). Altered firing rates may particularly impact neuropeptide release, which often requires higher level of neuronal activity (Nusbaum et al., 2001; van den Pol, 2012). Interestingly, enhancing or stimulating the rodent PFC suppresses cocaine-seeking behavior (Chen et al., 2013), and this effect may be due to normalizing astrocytic-maintained glutamate homeostasis (e.g., release and clearance) in the NAc (Berglind et al., 2009). In this latter study, intra-PFC microinjection of BDNF reversed cocaine-induced decreases in NAc extracellular glutamate levels, which have been largely attributed to Sxc activity. Hence, while it is known that cocaine-induced plasticity altering excitatory signaling involves changes in astrocytes and neurons in these regions, these collective findings support the possibility that cell-specific plasticity in the NAc may be causally related (e.g., due to a loss of PACAP). The potential for altered PACAP to contribute to cocaine-induced pathology is evident by our and others' studies showing that PACAP can regulate Sxc activity (Resch et al., 2014b; Kong et al., 2016), NMDAR and AMPAR signaling (Costa et al., 2009; Toda and Huganir, 2015), dendritic spine morphology (Gardoni et al., 2012), and LTP & LTD (Roberto and Brunelli, 2000) – all of which are altered in the NAc by cocaine.

Conclusion

Models indicate that incorporating neuronal-astrocyte communication increases the computational capacity of a neural network. Thus, the study of these forms of inter-cellular communication will be essential to our understanding of the local signal processing underlying normal and diseased brain functioning. Towards this, our work supports the idea that in the NAc, the presynaptic neuronal factor PACAP enables direct encoding of afferent information to astrocytes. This in turn promotes the activity of Sxc, a glutamate release mechanism that we found regulates AMPAR-mediated EPSCs in NAc efferents. Moreover, this integrated network is likely critical to the integration of the many inputs that are normally used to regulate adaptive behavioral responses. An interesting question is the degree to which this complex network resembles similar forms of integrated inter-cellular signaling in other systems or other regions of the brain. For example, it is shown that endocannabinoid neuromodulators activate CB1 receptors on hippocampal astrocytes (Navarrete and Araque, 2008). This leads to an increase in astrocytic glutamate release (presumed but not demonstrated to be a non-Sxc mechanism), which results in the activation of NMDARs on adjacent neurons (Navarrete and Araque, 2008). While there are interesting parallels to our own findings, this study highlights the potential for molecular integration through the release of postsynaptic factors (e.g. endocannabinoid). Hence, it is interesting to speculate that local processing may be dependent on incorporating the computational capacity of each cell in a given region, and this can be achieved by inter-cellular signaling originating from any of the participating cells. To the extent that this is the case, the key to developing a meaningful understanding

of CNS function in normal and disease states will require identifying the molecular basis for signal integration.

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