The Role of System XC- in Cognition: The Importance of Neuron-Astrocyte Signaling

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THE ROLE OF SYSTEM XC- IN COGNITION: THE IMPORTANCE OF NEURON-ASTROCYTE SIGNALING

by

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ABSTRACT
THE ROLE OF SYSTEM XC- IN COGNITION: THE IMPORTANCE OF NEURON-ASTROCYTE SIGNALING

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The biological basis of human intelligence is largely a mystery, but likely required evolutionary adaptations to achieve the information processing capacity needed to expand the complexity of cognition among species. The link between evolutionary expansion of signaling complexity in the brain and cognition has largely focused on neuronal mechanisms, in part because information processing has historically been attributed to these cells. However, astrocytes are emerging as a second type of brain cell that is capable of processing information due to their capacity to release glutamate and, thereby, regulate neural circuits. Hence, a modern question is whether astrocytes contributed to the signaling complexity required for sophisticated forms of cognition. The glutamate release mechanism system xc- (Sxc) is the ideal mechanism to investigate this question because it is evolutionarily novel to vertebrate species and it is expressed by astrocytes. The central hypothesis tested herein is that Sxc increased the complexity of glutamate signaling and is required for behavior requiring complex cognition. To test, a genetically modified rat with Sxc activity eliminated was generated (MSxc rats). Phenotyping revealed that loss of Sxc activity produced changes in behavior that reflect diminished cognition or top-down processing including impaired reversal learning, set-shifting, and attentional allocation. Remarkably, loss of Sxc did not impact central regulation of metabolism, Pavlovian conditioning, instrumental conditioning, locomotor activity, and novel-object recognition. Additionally, Sxc is integral to the regulation of neural networks. In the nucleus accumbens, we found that a loss of Sxc altered synaptic strength in a circuit specific manner. Further, we found that Sxc-mediated glutamate release is regulated by presynaptic (the neuropeptide PACAP), postsynaptic (endocannabinoid) and hormonal (glucocorticoids) signaling mechanisms. Further interrogation of Sxc regulation by PACAP revealed that this neuropeptide acts on both neurons and astrocytes to facilitate bidirectional neuron-astrocyte signaling between Sxc and extrasynaptic NMDA receptors. The in vivo relevance of this mechanism is established by our findings that PACAP microinjected into the nucleus accumbens attenuates cocaine-primed reinstatement, and the regulation of this behavior requires both Sxc and NMDA receptors. These findings support the possibility that future therapeutics could restore cognition by targeting astrocytes.

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CHAPTER I
THE BURDEN OF COGNITIVE DISORDERS AND THEIR CELLULAR AND MOLECULAR ORGINS

The Burden of Cognitive Disorders Represents an Unmet Medical Need

Neuroscience research has the potential to greatly reduce the burden of neurological disorders by discovering new molecular targets to replace the current therapeutic strategies that have failed to reduce disease burden. As of 2016, the World Health Organization (WHO) has established neurological disorders, including mental and behavioral disorders, as one of the leading causes of disease burden worldwide and in the United States as measured by disability-adjusted life years (DALYs) (Geneva, 2018). DALYs are a measure of the number of years of life lost to premature mortality and the years of life lost due to disability in the given population (Figure 1-1A). Of these neurological disorders, mental and behavioral disorders such as substance abuse, anxiety, depression, and schizophrenia contribute the greatest disease burden (Figure 1-1B). The high proportion of DALYs for mental and behavioral disorders is suggestive of a major medical need that is unmet by the current therapeutic strategies used. Given that the unmet medical need of CNS disorders has not decreased despite a more advanced understanding of the brain, a paradigm shift in both research focus and therapeutic strategy may be required.

Arguably, the most efficient strategy to reduce disease burden will involve researching and therapeutically targeting the most debilitating and common underlying symptoms that contribute to the etiology of mental and behavioral

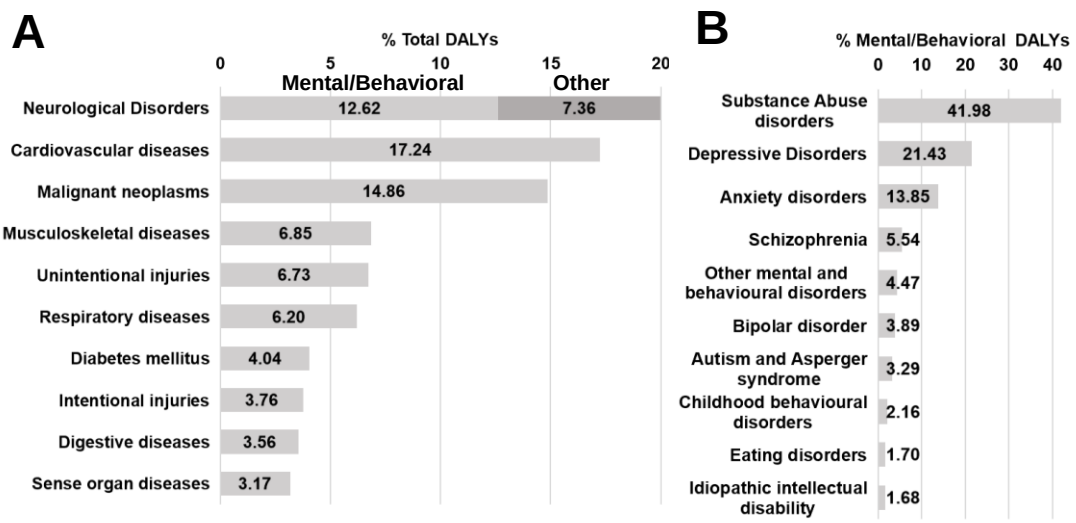


Figure 1-1: WHO 2016 percent of total disease-adjusted life years (DALYs) lost to major diseases in the United States of America. A, Data was tabulated from the 2016 WHO estimates for the top 10 diseases with the greatest life burden. **B,** Data depicts proportion of DALYs under the mental/behavioral disorder category. Substance abuse disorders include opioid and alcohol use.

disorders. This ideology is supported by a recent change in research philosophy established by the National Institute of Mental Health called Research Domain Criteria (RDoC). One of the principle aims of RDoC is to identify the fundamental and discrete components underlying multiple disorders that can be readily studied by researchers with the goal of more efficiently reducing disease burden (Cuthbert & Insel, 2013). This is in contrast to canonical approaches where research focus was aimed towards studying the underlying basis of multi-faceted diseases such as depression or schizophrenia which are exceptionally difficult to model in rodents. Given this, RDoC has the potential to transform neuroscience research and therapeutic strategies through revealing the neurobiological basis of discrete brain functions (e.g., cognitive control) which could lead to improved patient care for multiple diseases that share a particular symptom.

Substance abuse, depression, anxiety, and schizophrenia all share a debilitating common symptom, cognitive dysfunction (Millan et al., 2012). Cognition represents a complex array of discrete forms of brain function ranging from simple forms of learning and memory to much more complex forms that are often collectively referred to as executive functions such as self-control and selective attention (Diamond, 2013; MacLean, 2016; Ralph, Jefferies, Patterson, & Rogers, 2017). As such, cognition is a type of brain function that is highly amendable to an RDoC approach in that researchers can focus on discrete cognitive processes (e.g. self-control) that may benefit multiple disorders. Thus, if we can understand the cellular and molecular mechanisms underlying discrete cognitive processes in the brain, we can develop improved therapies and significantly reduce the disease burden of cognitive disorders. Unfortunately, uncovering the basis of cognition is complicated by the brain being an exceptionally complex biological system which has stymied the emergence of successful therapeutics (Millan et al., 2012).

Establishing the Importance of Glutamate and Astrocytes to Cognition

Our incomplete understanding of the human brain and more specifically intercellular communication is a fundamental barrier in the development of disease modifying therapeutics that are needed to mitigate the impact of cognitive disorders (Sundstrom, 2007). Central to this endeavor is thoroughly revealing the molecular, cellular, and structural components of intercellular communication which collectively dictate how information is transferred from cell

to cell within neural circuits; especially since neurotransmitter receptors are the most common target for CNS therapeutics (Raiteri, 2006).

Due to the mathematical complexity created by billions of cells communicating across trillions of synapses, the analysis of nervous system evolution may provide useful insights into the organizational and signaling principles of the vertebrate brain that engendered enhanced cognitive abilities which may allow us to refine our therapeutic targets (Bosch et al., 2017; Emes et al., 2008; Herculano-Houzel, 2012; Hoshiba, Wada, & Hayashi-Takagi, 2017; Tang, Nyengaard, De Groot, & Gundersen, 2001). At the molecular level, phylogenetic studies have detected several classical neurotransmitters present in vertebrates such as glutamate, gamma-aminobutyric acid (GABA), histamine, and monoamines in at least some of the basal metazoan clades which suggests each of these molecules were essential components during the evolution of the nervous system (Moroz, 2009, 2015; Moroz & Kohn, 2016; J. F. Ryan & Chiodin, 2015; J. F. Ryan et al., 2013). However, the amino acid neurotransmitters glutamate and GABA are unique relative to histamine and monoamines. First, these neurotransmitters often *directly* activate ligand-gated ion channels to produce rapid changes in the excitation state of neurons. Second, glutamate and GABA are expressed in most if not all neural circuits in the brain. Because of this, glutamate and GABA are often modeled as the primary excitatory and inhibitory neurotransmitters, respectively, and are involved in most forms of information transfer in vertebrate nervous systems (Goetz, Arslan, Wisden, & Wulff, 2007; Traynelis et al., 2010). In further support, disrupted glutamate signaling has been

linked to cognitive dysfunction seen in schizophrenia, bipolar disorder, depression and substance abuse and has therefore driven research focus towards therapies that target glutamate receptors and transporters (Dauvermann, Lee, & Dawson, 2017; Kalivas, 2009; C.-T. Li, Yang, & Lin, 2019). Thus, as it relates to intercellular communication, information transfer and cognitive processing, understanding the forces influencing glutamate signaling will be essential to any endeavor seeking to resolve how and why cognitive disorders emerge.

Evolutionary adaptations to nervous systems enabling increased computational capacity of cellular networks and improved energy efficiency were likely crucial to phylogenetic gains in cognitive ability (Bosch et al., 2017; Emes et al., 2008; Niven & Laughlin, 2008; T. J. Ryan & Grant, 2009; Yu & Yu, 2017). For example, a key evolutionary event in the expansion of intercellular signaling in complex nervous systems was the recruitment of astrocytes to the processing, integration, and storage of information in conjunction with providing needed metabolic and structural support to neural networks (Alvarellos-Gonzalez, Pazos, & Porto-Pazos, 2012; Min, Santello, & Nevian, 2012; Oberheim, Wang, Goldman, & Nedergaard, 2006; Porto-Pazos et al., 2011). To achieve this, astrocytes are capable of monitoring the activity of thousands to millions of synapses due to the expression of most of the traditional neurotransmitter receptors and are capable of integrating this information and communicating back to neurons through the release of a variety of neuroactive substances (Bormann & Kettenmann, 1988; Cahoy et al., 2008; Clarke & Barres, 2013; Glaum, Holzwarth, & Miller, 1990;

Grybko, Sharma, & Vijayaraghavan, 2010; Navarrete & Araque, 2010; Oberheim et al., 2006; Ogata & Kosaka, 2002; Pruss, Akeson, Racke, & Wilburn, 1991; Shen & Yakel, 2012; von Blankenfeld & Kettenmann, 1991; Zanassi, Paolillo, Montecucco, Avvedimento, & Schinelli, 1999). Importantly, astrocytes can leverage glutamate as a signaling molecule to regulate neural networks while also using it as a metabolic substrate to provide energy for high intensity information processing. As such, glutamatergic signaling by astrocytes should be seriously considered for their potential role in cognition.

Intriguingly, the phylogenetic increases in the complexity of central nervous systems was accompanied by dramatic changes in astrocytes or glial cells. For example, *C. elegans* expresses 0.2 glia for every neuron, whereas the brain of *H. sapiens* has 1.4 glia for every neuron (Nedergaard, Ransom, & Goldman, 2003). As a result, glia are the most abundant cell type in the human brain (Herculano-Houzel, 2012). Remarkably, the evolution of astrocytes occurred on a pace rapid enough that astrocytes can even be distinguished between closely related species. For example, human cortical astrocytes are reported to be 2.6-fold larger in diameter, extend 10-fold more primary processes, and propagate intracellular signals (i.e., Ca^{2+} waves) four-fold faster than rodent astrocytes. The human brain even has anatomically-defined subclasses of astrocytes that are not found in rodents (Oberheim et al., 2009). Hence, there is a fundamental need to understand how glutamate signaling between neurons and astrocytes regulates the function of complex brains

(Alvarellos-Gonzalez et al., 2012; Min & Nevian, 2012; Oberheim et al., 2009; Oberheim et al., 2006).

Over the past few decades, astrocytes have been found to contribute to virtually every aspect of brain function, including neural development, synapse formation, synapse pruning, synaptic plasticity, glutamate signaling, gating of neural circuits for excellent reviews (Allen & Barres, 2005; Christopherson et al., 2005; Haydon, Blendy, Moss, & Rob Jackson, 2009; Oberheim et al., 2006; Papouin, Dunphy, Tolman, Foley, & Haydon, 2017; Perea, Sur, & Araque, 2014; Porto-Pazos et al., 2011; Poskanzer & Yuste, 2011). Despite the broad functional role of these cells, the potential for these cells to underlie cognition is a new concept (Santello, Toni, & Volterra, 2019). The following sections will discuss the functional relationships between astrocytes and neurons and how they rely on glutamate for information transfer and how glutamate signaling from these cells may impact the information processing capacity of neural circuits and consequently impact cognition. Figure 1-2 represents some of the key components of glutamate signaling within the glutamate synapse. This model is meant to set the framework for what is known about glutamatergic signaling and set the context for the experiments and data presented in the following chapters.

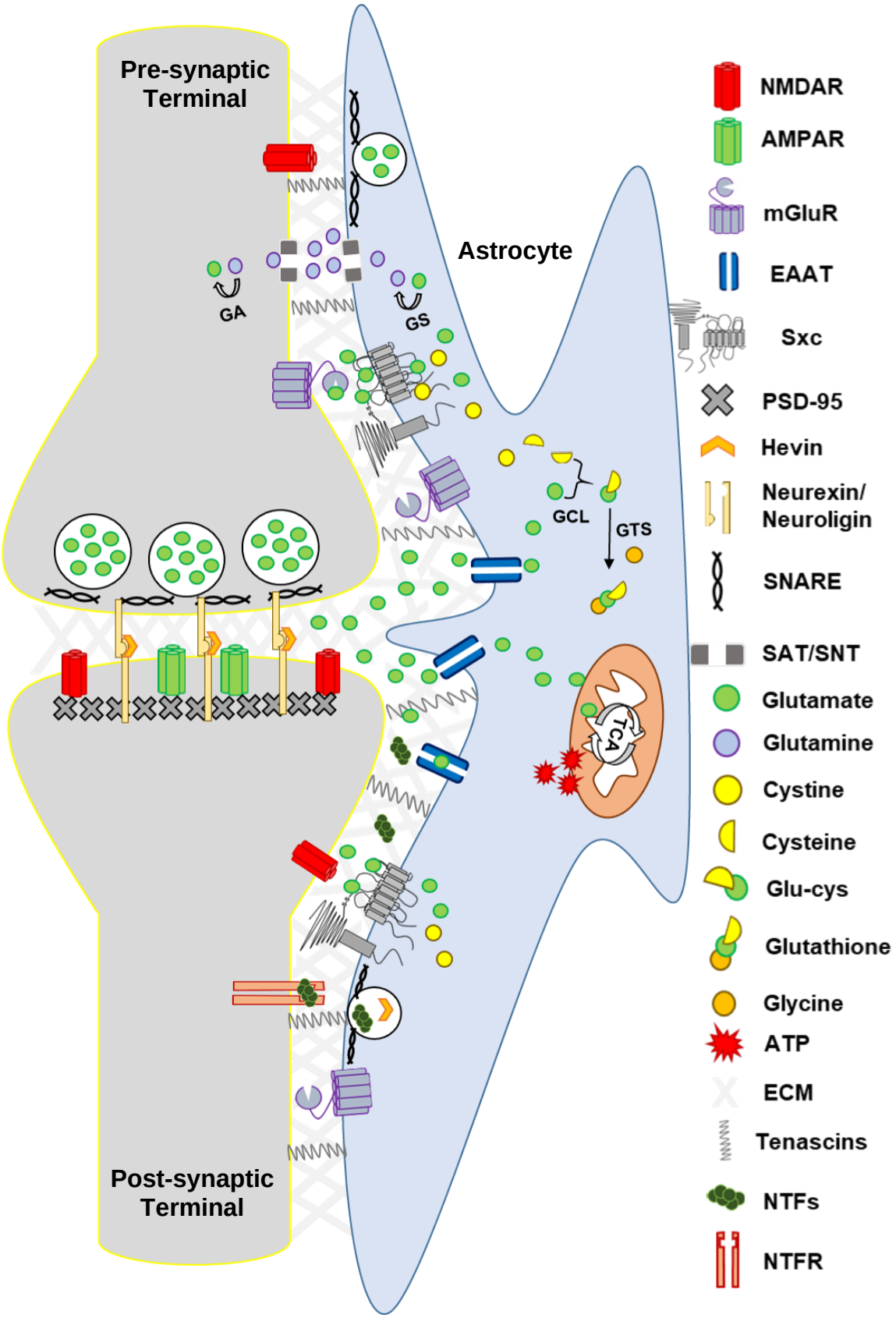

Pre-synaptic Terminal
Post-synaptic Terminal
Astrocyte
GA
GS
GCL
GTS
TCA
NMDAR
AMPAR
mGluR
EAAT
Sxc
PSD-95
Hevin
Neurexin/ Neuroligin
SNARE
SAT/SNT
Glutamate
Glutamine
Cystine
Cysteine
Glu-cys
Glutathione
Glycine
ATP
ECM
Tenascins
NTFs
NTFR

Figure 1-2: Model for glutamate homeostasis within the tri-partite synapse. The cellular components of the tri-partite are illustrated by the pre- and post-synaptic terminals and the peri-synaptic astrocytic process. Key glutamate enzymes include glutaminase (GA) which converts glutamine to glutamate within the pre-synaptic terminal, and glutamine synthetase (GS) which converts glutamate to glutamine within astrocytes. Glutamate-cysteine ligase (GCL) dimerizes glutamate and cysteine (reduced cystine) and undergoes an additional enzymatic step via glutathione synthetase (GTS) to produce glutathione. Glutamate can also be converted to α-ketoglutarate and re-enter the TCA cycle to produce ATP. Vesicular release mediated by the SNARE complex, excitatory amino acid transporters (EAATs), and the cystine-glutamate antiporter system x_c^- (Sxc) help maintain intra and extracellular glutamate balance. Glutamate receptors such as AMPAR, NMDAR, and metabotropic glutamate (mGluR) receptors exist within the synaptic and extrasynaptic space and are activated by different sources of glutamate. Neurotrophic factors (NTFs) are released by astrocytes and signal through their receptors (NTFRs) on neurons to facilitate glutamate receptor expression. The foundation for the structural basis of the tripartite synapse is the extracellular matrix (ECM). The volume of space between astrocytes and neurons are dictated by tenascins, which is critical for the diffusion of glutamate and its ability to access extrasynaptic receptors. The synapse is stabilized by neurexins and neuroligins which bridge the pre-synaptic SNARE complex and the postsynaptic density (PSD-95) to ensure efficient chemo-electrical transfer of information. This is further stabilized by proteins released by astrocytes such as hevin.

A Brief Overview of Glutamate Metabolism in the Tripartite

Glutamate may have evolved into the primary excitatory neurotransmitter in the brain because it is highly abundant and couples key cellular processes such as energy and antioxidant production needed for the efficient expansion of nervous systems (Moroz & Kohn, 2016).

Astrocytes serve as the major source of metabolically derived glutamate in the brain and have an efficient network of transporters and enzymes to tightly regulate glutamate concentration, metabolism, as well as providing glutamate precursors to neurons (Schousboe, Scafidi, Bak, Waagepetersen, & McKenna, 2014). The canonical glutamate synthesis pathway within the astrocyte involves the synthesis of pyruvate from glycolysis which enters the tri-carboxylic acid (TCA) cycle to produce α-ketoglutarate (α-KG). Other than continuing through the cycle, α-KG can undergo reversible transamination catalyzed by glutamate

dehydrogenase to produce glutamate. Glutamate can then be converted into glutamine via the astrocyte specific enzyme glutamine synthetase. Glutamine is subsequently exported from the cell via system N transport (SN1) where it enters the extrasynaptic space awaiting import into presynaptic nerve terminals via system A transport (SAT). The accumulated glutamine within the presynaptic terminal is converted back into glutamate via glutaminase which can then be packaged into vesicles and released into the synaptic cleft. To complete the cycle, released glutamate is taken up by sodium dependent astrocytic glutamate transporters such as GLT-1. Studies predict that about 85% of the re-accumulated glutamate within the astrocytic cytosol is converted back into glutamine to be released again, with the rest undergoing deamination back into α-KG which re-enters the TCA cycle to be recycled for further ATP production (Rothman, De Feyter, de Graaf, Mason, & Behar, 2011). This outline of the glutamate-glutamine cycle highlights two major roles of glutamate in the synergistic relationship between neurons and astrocytes within the peri-synaptic space; glutamate is traded as a metabolic molecule in addition to acting as a signaling molecule.

The conversion of glutamate back into α-KG to re-enter the TCA cycle and drive ATP production demonstrates its role as a key metabolic currency. The ratio of glutamate that is shunted back through the TCA cycle to be oxidized for ATP production is dynamically regulated depending on the state of the external milieu and internal energy demand of the astrocyte. For example, exogenously applied glutamate to astrocyte cultures is converted back into α-KG which in a

stepwise manner produces malate then pyruvate which can re-enter the TCA cycle as acetyl-CoA (McKenna, 2013). The rate of this phenomenon is determined by the extracellular concentration of glutamate. In support, models estimate glutamate to have a peak concentration of 1.5 mM following quantal release within the synapse with transient extracellular glutamate at about 10 μM (Matsui, Jahr, & Rubio, 2005). When glutamate is exogenously applied within this range (100 to 500 μM) the astrocyte responds by shuttling a larger proportion of glutamate through this oxidative pathway; an increase from about 15.3% at baseline to 42.7%. As a result, less glutamate is converted back into glutamine in astrocytes which reduces available glutamine for neurons from 85% at baseline to 57% (McKenna, Sonnewald, Huang, Stevenson, & Zielke, 1996). The use of glutamate as an energy source is theorized to result from the intense energy demand within astrocytes. Specifically, the increase in extracellular glutamate following phasic neuronal release requires uptake of glutamate via sodium-dependent astrocyte transporters e.g. GLT-1. This produces a significant sodium gradient that requires the utilization of ATP to be corrected via sodium/potassium ATPase (McKenna, 2013).

Models of glutamate release and clearance demonstrate how efficient glutamate signaling is in that it has the potential to produce more energy than it spends. In support, one quanta of glutamate amounts to about 4000 molecules (Riveros, Fiedler, Lagos, Munoz, & Orrego, 1986) with one quanta being released per action potential according to estimates in hippocampal neurons (T. A. Ryan & Smith, 1995). This would require 12000 Na^+ ions to be extruded

following uptake via GLT-1 which amounts to 4000 Na^+/K^+ ATPase cycles and an equivalent number of ATP molecules required to correct the Na^+ gradient. Yet, the net energy yield for the uptake and oxidation of 1 glutamate molecule is about 23-26 ATP; theoretically about 160 glutamate molecules would be sufficient to pay for the uptake of 4000 molecules (McKenna, 2013). These estimates predict glutamate uptake and oxidation is providing a net excess of ATP. Hence, neuron-astrocyte glutamate signaling is highly efficient and produces a net excess of energy which can fuel the processing power of neural networks required for cognition.

The abundance of glutamate may also be attributed to its use as a substrate for the brain anti-oxidant glutathione (Yelamanchi et al., 2016). Importantly, glutamate signaling can pose serious challenges to a nervous system due to the metabolic demand it creates and the risk of excitotoxicity (Lewerenz & Maher, 2015). In order to counteract this risk, neural cells adapted a mechanism to utilize excess glutamate in conjunction with cysteine and glycine to produce glutathione. Glutathione is synthesized within astrocytes via a series of enzymatic reactions. First, the enzyme glutamate-cysteine ligase (GCL) catalyzes the linking of glutamate and cysteine. Next, glutamate-cysteine is linked to glycine via the actions of glutathione synthetase (GS) to produce glutathione (Bridges, Lutgen, Lobner, & Baker, 2012). Because of the involvement of glutamate in the citric acid cycle and biosynthesis of the antioxidant glutathione, the selection of glutamate as the primary excitatory neurotransmitter may have helped address the balance of excitotoxicity and

oxidative stress by efficiently coupling excitatory signaling with energy production and oxidative stress defense mechanisms.

Given these metabolic mechanisms, glutamate was an ideal molecule to evolve into the single most abundant amino acid and the primary excitatory signal in the vertebrate brain (Abeles, 1991; Braitenberg, Schüz, & Braitenberg, 1998; Kasthuri et al., 2015; Schousboe, 1981).

Glutamate Signaling in the Synaptic Cleft

The glutamate synapse represents the most common functional domain in the brain, with estimates placing glutamate at nearly 95% of cortical synapses (Kasthuri et al., 2015). The typical glutamate synapse is comprised of a pre-synaptic terminal, a postsynaptic terminal, and a perisynaptic astrocytic process (as depicted Figure 1-2). This section will consider the factors that influence each of these components and how they work together in order to efficiently transfer information.

The release of glutamate from the presynaptic neuron occurs in the active zone, a specialized region in axonal terminals or boutons containing hundreds of proteins that collectively create a highly organized matrix enabling calcium-dependent release of glutamate (Gronborg et al., 2010; Korber & Kuner, 2016; Ribrault, Sekimoto, & Triller, 2011). Ca^{2+}-dependent release involves a series of highly orchestrated events that results in the formation of a fusion pore that can be shared between the plasma membrane and the membranes of synaptic vesicles containing high mM levels of glutamate (Burger et al., 1989; Shupliakov, Brodin, Cullheim, Ottersen, & Storm-Mathisen, 1992). The release probability of

glutamate is influenced by Ca^{2+} influx into the presynaptic terminal through voltage-gated calcium channels (VGCCs) which are triggered by the arrival of action potentials (Branco & Staras, 2009). Ca^{2+} is received by synaptotagmin, which are a class of trans-vesicular membrane associated proteins belonging to the SNARE complex that drive vesicular docking, fusion, and release of glutamate (Südhof, 2013).

There are multiple presynaptic glutamate receptors that may enable regulation over the pattern and amount of presynaptic glutamate release that impact Ca^{2+} dynamics in the terminal. First, the metabotropic glutamate receptors (mGluRs) which belong to the G-protein coupled receptor (GPCR) family can impact pre-synaptic glutamate release. There are three major classes of mGluRs; group 1,2, and 3 which have varying cellular expression and localization. The predominant mGluRs expressed in the pre-synaptic terminal belong to group 2 and 3. These groups signal through G_i/G_o which inhibit adenylate cyclase and consequently ion channels such as VGCCs (Niswender & Conn, 2010). For example, application of a group 2 mGluR agonist to hippocampal slices reduced pre-synaptic Ca^{2+} influx which produced a reduction in mEPSC frequency at the mossy fiber-CA3 synapse (Kamiya & Ozawa, 1999).

Ionotropic glutamate receptors may also contribute to pre-synaptic regulation of synaptic glutamate release. The two most common ionotropic glutamate receptors are n-methyl d-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). NMDARs and AMPARs are characterized as tetramers, containing four subunits with a

central ion pore. Their principle function is to conduct ionic currents in order to rapidly depolarize neurons or trigger intracellular signaling cascades (Traynelis et al., 2010). In the presynaptic compartment, NMDARs are largely involved in positively influencing neurotransmitter release. Their effect can either be direct or indirect in that activation can directly increase Ca^{2+} influx into the terminal and promote vesicular fusion. Conversely, activation can cause membrane depolarization that is sufficient to trigger adjacent VGCCs which indirectly increases Ca^{2+} influx. Regardless of the mechanism, pre-synaptic NMDARs have been shown to influence spontaneous and evoked glutamate release (Banerjee, Larsen, Philpot, & Paulsen, 2016). Conversely, there is little evidence for pre-synaptic AMPARs, as their presynaptic expression appears to be brain region dependent (Rossi, Maton, & Collin, 2008).

Estimates suggest that each action potential releases one quanta of glutamate that amounts to about 4000 molecules. Other estimations establish a range of 3,000-10,000 molecules of glutamate following each action potential (Barbour & Hausser, 1997; Clements, 1996; Pendyam, Mohan, Kalivas, & Nair, 2009). Regardless, this is sufficient to increase extracellular glutamate concentration from the nM to the low mM range (0.5-1.0 mM) but only in the aspect of the synaptic cleft that is most proximal to the release sites. This results in a microdomain of high glutamate concentration that approximates the affinity of AMPARs for glutamate (Rusakov, 2001).

The distribution of glutamate receptors on the post-synaptic terminal is highly organized reflecting, in part, relative affinities for glutamate. Efficient

signaling between the pre and postsynaptic neuron is influenced by the spatial distribution of low-affinity AMPAR which are expressed on the closest aspect of the post-synaptic membrane relative to the release sites and high-affinity NMDAR and mGluRs being expressed more laterally. The localization of glutamate receptors is largely driven by a transcellular protein bridge created by neurexins, neuroligins and matricellular proteins that link the pre-synaptic release machinery (SNARE complex) to the post synaptic density (PSD) that anchors AMPARs and NMDARs (Christopherson et al., 2005; Eroglu et al., 2009; Hata, Davletov, Petrenko, Jahn, & Südhof, 1993; Kania & Klein, 2016; Kucukdereli et al., 2011). The transcellular protein bridge is significant because it ensures the intimate localization of glutamate release sites with the post-synaptic terminal, which makes synaptic signaling highly efficient. This level of efficiency is required, in part because an estimated 50-90% of synaptic glutamate molecules exit the cleft within 10-70 ms (Savtchenko & Rusakov, 2004). Thus, the concentration of glutamate falls rapidly across temporal and spatial dimensions. Activation of low-affinity AMPARs is more sensitive to spatiotemporal regulation than high-affinity glutamate receptors such that lateral trafficking of AMPARs represents a key form of synaptic plasticity influencing sensitization (for review see, Rusakov, Savtchenko, Zheng, & Henley, 2011). Indeed, when AMPARs are experimentally immobilized, they rapidly desensitize in response to glutamate application which greatly reduces post-synaptic currents (Heine et al., 2008). As a result, AMPARs are ideal targets for signaling mechanisms to exert powerful control over the strength of synaptic transmission.

While synaptic AMPARs function to rapidly transmit glutamate signals, the function of synaptic NMDARs and mGluRs tend to be slower and are meant to augment the function of AMPARs. Importantly, NMDARs are under a unique form of regulation relative to AMPARs. Instead of regulation linked to glutamate affinity and diffusion, a major regulatory mechanism for NMDARs is the voltage-dependent physical occlusion of their ion pore by a Mg^{2+} ion. In order for this block to be lifted, sufficient membrane depolarization via AMPARs is required (Traynelis et al., 2010). Once lifted, NMDARs can conduct Ca^{2+} ions which can activate a variety of intracellular signaling cascades that activate kinases and trigger the synthesis of new proteins. Synaptic mGluRs synergize these effects by also contributing to the propagation of Ca^{2+}-dependent effects. Broadly, the impact of synaptic NMDARs and mGluRs tend to stabilize the synaptic structure and boost AMPAR-mediated signaling in a phenomenon referred to as long-term potentiation LTP (Bliss & Collingridge, 1993). Thus, post-synaptic AMPARs, NMDARs, and mGluRs synergize to effectively encode pre-synaptic glutamate signals.

While diffusion of glutamate from the synapse represents an important phenomenon for the regulation of synaptic signaling, the last component of the synapse, the astrocytic process, is involved in synaptic glutamate clearance. It has been theorized that for every molecule of glutamate released, roughly 4000 molecules per quanta, there is an equal number of transporters ready to remove glutamate from the cleft. Specifically, the excitatory amino acid transporter (EAAT) GLT-1, the most abundant astrocytic glutamate transporter in the brain,

assumes the role as the primary glutamate clearance mechanism (Lehre & Danbolt, 1998). Intriguingly, recent findings have demonstrated that GLT-1 is a highly mobile protein in astrocyte membranes which is functionally relevant to controlling glutamate within the synapse. Under periods of low neuronal activity, GLT-1 is anchored at the synapse waiting for glutamate to be released. As glutamate concentrations climb, GLT-1 binds glutamate and rapidly diffuses out of the synapse allowing for unbound transporters to rotate in. Interestingly, when GLT-1 is experimentally immobilized, its ability to clear glutamate is not impaired but post-synaptic currents are increased, suggesting that GLT-1 has a dual function in not only transporting glutamate into the astrocyte, but also moving it away from low affinity AMPARs and into the extrasynaptic compartment (Murphy-Royal et al., 2015). Thus GLT-1 is important for maintaining the sensitivity of AMPARs within the synapse and controlling the diffusion of glutamate out of the synapse. Thus, the function of the peri-synaptic astrocytic process demonstrates how important astrocytes are in the regulation of synaptic glutamate signaling.

The basic elements of glutamate's actions in the synaptic cleft involving the activity-dependent release, diffusion across a synaptic cleft, and activation of postsynaptic receptors resembles what has been in place throughout much of the evolution of metazoan nervous systems. In other words, synaptic glutamate is among the most ancient of signaling processes (Moroz, 2009, 2015; Moroz & Kohn, 2016; J. F. Ryan & Chiodin, 2015; J. F. Ryan et al., 2013). However, nervous systems have expanded upon this essential unit of the nervous system by increasing the complexity of intercellular communication through establishing

a network of glutamate receptors, transporters, and release mechanisms distributed outside of the synapse on both neurons and astrocytes.

Glutamate Signaling in the Extrasynaptic Space

The opportunity to increase the complexity of signaling in the brain likely required extrasynaptic signaling. Estimates suggest that the synaptic cleft represents 2% or less of the total interstitial space (Rusakov, Harrison, & Stewart, 1998). Evolution has equipped the remaining 98% of interstitial space with glutamate receptors, transporters, and release mechanisms that are expressed throughout the full extent of neuronal and astrocytic cellular membranes, which provides the molecular basis for the diverse forms of signaling. Importantly, the extrasynaptic compartment conveys unique forms of information transfer relative to the synapse. As a result, synaptic and extrasynaptic glutamate signaling occurring between neurons or between neurons and astrocytes can be segregated into functionally distinct, specialized signaling zones or microdomains (Dityatev, Seidenbecher, & Schachner, 2010; Le Meur, Galante, Angulo, & Audinat, 2007; Malarkey & Parpura, 2008; Moussawi, Riegel, Nair, & Kalivas, 2011; Papouin & Oliet, 2014; Wu, Grebenyuk, McHugh, Rusakov, & Semyanov, 2012). Given the massive surface area of the extrasynaptic compartment, it is physically created by the two most abundant cells in the brain: astrocytes and neurons. Of particular interest to this work is the contribution of astrocytes to the extrasynaptic space which includes monitoring neuronal activity, integrating neuronal information, and transforming that information into a functional output to influence the activity of entire neural

networks. Astrocytes achieve this through the reception and release of a variety of neuroactive substances such as glutamate and trophic factors (e.g., BDNF, TGF-β) (Verkhratsky, Matteoli, Parpura, Mothet, & Zorec, 2016).

Regarding how astrocytes can monitor and integrate neuronal information, glutamate that enters the extrasynaptic compartment can gain access to receptors present on astrocytes which are largely thought to be mGluRs. Notably, the expression of functional ionotropic receptors in astrocytes (primarily NMDARs and AMPARs) varies greatly depending on brain region and experimental conditions and will not be discussed here (Hoft, Griemsmann, Seifert, & Steinhauser, 2014). The two most predominant mGluRs expressed by astrocytes are mGluR5 and mGluR3 (Bradley & Challiss, 2012; Schools & Kimelberg, 1999) which belong to group 1 and group 2 respectively. MGluR5 activation by glutamate triggers phospholipase C activity which produces inositol triphosphate (IP_3) and diacylglycerol (DAG) resulting in Ca^{2+} release from the endoplasmic reticulum. Intriguingly, the Ca^{2+} response oscillates with mGluR5 activation, which is dependent on a PKC mediated negative feedback loop which momentarily phosphorylates mGluR5 and decouples it from $G\alpha_q$. Importantly, increasing glutamate concentrations has no effect on the frequency or amplitude of Ca^{2+} oscillations once the signaling loop has been initiated. In a sense, this cascade is “all or none” and simply encodes the presence of sufficient activity from adjacent neurons. The functional consequence of mGluR5 activation and these Ca^{2+} oscillations is still under investigation, but one of the current theories suggests that activation of mGluR5 enhances glutamate uptake into the astrocyte

via increasing GLT-1 expression which facilitates clearance of extracellular glutamate (Devaraju, Sun, Myers, Lauderdale, & Fiacco, 2013; Umpierre, West, White, & Wilcox, 2019; Vermeiren et al., 2005).

MGluR3, while having a different G-protein coupling mechanism, has some similarities in function with mGluR5 in astrocytes. Activation of mGluR3 has been shown to produce enhancements in glutamate transporter expression (Aronica et al., 2003), which suggests that the astrocytic mGluRs serve redundant functions in receiving qualitative information about nearby neuronal activity and consequently working to reduce the concentration of extracellular glutamate. Thus, activation of either receptor can serve as a neuroprotective mechanism against glutamate induced excitotoxicity. For example, mGluR3 has been shown to increase the production and release of trophic factors such as TGF-β and NGF which can act synergistically on adjacent neurons to promote survival (Bruno et al., 1998; Ciccarelli et al., 1999). Additionally, mGluR5 facilitates neuroprotective effects via astrocytes in a model of ischemic stroke (Cavallo et al., 2020) With both the actions of both mGluR3 and 5 in astrocytes considered, the net effect of glutamate acting on these receptors appears to be maintaining extracellular glutamate levels within a safe range while at the same time providing neuroprotective factors.

An emerging principle of brain function is that astrocytes can actively release glutamate to regulate neurons (Baker, Xi, Shen, Swanson, & Kalivas, 2002; Jourdain et al., 2007; Martin, Bajo-Graneras, Moratalla, Perea, & Araque, 2015). Notably, the actions of glutamate released by astrocytes can be

functionally distinct from neuronally derived glutamate due to spatial and temporal receptor activation parameters. In other words, astrocytes may target glutamate release to a population of neuronal receptors that are distal from synaptic release sites. These receptors are often embedded in large protein complexes and have different functions relative to their synaptic counterparts. For example, extrasynaptic NMDARs have been detected in highly-specialized protein complexes that are often anchored near adjacent astrocyte processes (Petralia et al., 2010; Sah, Hestrin, & Nicoll, 1989), and extrasynaptic NMDARs can have effects on the neuron that are opposite to synaptic NMDAR activation such as producing an attenuation rather than enhancement of excitatory output (Papouin & Oliet, 2014). Thus, understanding the factors regulating astrocytic release of glutamate will be essential to understanding how astrocytes can impact neural circuits and consequently behavior.

The mechanisms of glutamate release from astrocytes fall into two major groups: channel/transporter mediated and vesicular mediated (Malarkey & Parpura, 2008). For example, the cystine-glutamate antiporter system x_c^- (Sxc) which represents a non-vesicular release mechanism, has been shown to release glutamate that can activate pre and post-synaptic glutamate receptors in the striatum and hippocampus to regulate synaptic strength (Baker et al., 2002; Lewerenz et al., 2013; Williams & Featherstone, 2014). Similarly, ultrastructural electron microscopy studies provide evidence for vesicles containing glutamate in hippocampal astrocytes proximal to pre-synaptic neuronal NMDARs which when activated enhance synaptic glutamate release and strength (Bezzi et al.,

2004; Jourdain et al., 2007; Xu et al., 2007). Furthermore, TREK-1 and Best1, which are both glutamate-permeable channels, serve as fast/phasic and slow/tonic mechanisms of glutamate release from astrocytes (Woo et al., 2012). The significance of their differing transport velocities is revealed by their respective activation of neuronal mGluRs and NMDARs which consequently tune synaptic transmission. Given the variety of release mechanisms, a question that needs to be resolved is under what specific circumstances do each of these mechanisms become active and how might they be linked to or disrupted in disease?

A common underlying factor that enhances glutamate release from astrocytes is elevations in astrocytic intracellular Ca^{2+}, which might explain how some glutamate release mechanisms in astrocytes become active. As is the case in neurons, Ca^{2+} is the key signal that mediates the fusion of vesicles with the membrane via the SNARE complex, so it is likely that astrocytes have a similar mechanism so long as the machinery is present. Indeed, electron microscopy studies have shown the vesicular glutamate transporter VGLUT1/2 and SNARE protein cellubrevin in hippocampal astrocytes (Bezzi et al., 2004). However, the relevance of these findings to *in vivo* function remains a controversial topic. In particular, the use of genetically modified mice designed to lack vesicular release machinery in astrocytes in experiments seeking to investigate its role in regulating neural networks *in vivo* revealed transgene expression in neurons which quelled of the relevance of the findings (Bohmbach, Schwarz, Schoch, & Henneberger, 2018; Fujita et al., 2014). However, even without vesicular release

machinery, Ca^{2+} can also impact transporter-mediated glutamate release such as through Best1 (Sun, Tsunenari, Yau, & Nathans, 2002). Likewise, volume-regulated anion channels (VRACs) are opened during astrocytic swelling which can release excitatory amino acids such as glutamate (Kimelberg, Goderie, Higman, Pang, & Waniewski, 1990). VRACs do appear to be somewhat sensitive to Ca^{2+}, as Ca^{2+}-dependent astrocyte swelling stimulated their opening. While it is unknown whether other transport mechanisms have a documented Ca^{2+} sensitivity, it is certainly possible that Ca^{2+} dependent kinases may regulate transporter activity through phosphorylation. More work is needed to explore this possibility.

Glutamate release from astrocytes can occur even in the absence of Ca^{2+} signaling. For example, the purinergic receptor P2X7 is a ligand-gated ion channel activated by ATP that is capable of releasing glutamate and is not negatively impacted by a lack of Ca^{2+} (Duan et al., 2003). Additionally, the connexin 43 hemichannels, which are capable of transporting glutamate, glutathione, ATP, as well as cations do not appear to be activated by Ca^{2+}, rather they serve an important role in balancing Ca^{2+}, among other molecules, across the cell membrane (Liang et al., 2020).

Considering the mechanisms of glutamate release from astrocytes and the power that they have in regulating neurons, lingering questions arise regarding how these components impact cognitive processing and behavior. An emerging viewpoint of the field, and the one that will be established by the experiments in the following chapters is that neurons and astrocytes work together to dictate

how glutamate engages its receptors to regulate neural networks and cognition. *The overarching question that will be answered by these experiments is whether astrocytes were involved in the expansion of cognitive abilities in vertebrates due to their ability to process and disseminate information through glutamate. Ultimately, do these cells contain the glutamatergic components that enhanced our cognitive abilities that when disrupted make us vulnerable to cognitive disorders?*

CHAPTER II
THE ROLE OF SYSTEM XC- IN COGNITION AND THE REGULATION OF NEURAL CIRCUITS

INTRODUCTION

Cognitive deficits contribute to the disease burden associated with numerous disorders including mental health, cancer, diabetes, heart disease, and obesity (Cannon et al., 2017; Harrison & Wefel, 2018; He, Ryder, Li, Liu, & Zhu, 2018; Jones, Gray, & Hemsley, 1992; Lange, Seer, & Kopp, 2017; P. M. Moran, Owen, Crookes, Al-Uzri, & Reveley, 2008; Rock, Roiser, Riedel, & Blackwell, 2014; Y. Yang, Shields, Guo, & Liu, 2018). Mental health disorders in particular such as depression and substance abuse register as leading causes of disease burden in high-income countries (DiLuca & Olesen, 2014; Gustavsson et al., 2011; Lassek, Weingarten, & Volknandt, 2015; Olesen & Leonardi, 2003; WHO, 2008). Considering this, resolving cognitive dysfunction would greatly reduce disease burden. While this renders the development of safe, effective cognitive pharmacotherapies as a high priority, there has been insufficient progress due to critical gaps in our understanding of the cellular and molecular basis of cognition in the brain. An emerging viewpoint is that the molecular basis of cognition in vertebrates likely arose from the evolutionary enhancement of signaling complexity in the brain (Emes et al., 2008; Nithianantharajah et al., 2013), which may support novel strategies to understand and treat cognitive dysfunction.

The evolutionary expansion of glutamate neurotransmission contributed to the signaling complexity needed for the progression of cognitive ability. For

example, synaptic glutamate became an important form of intercellular signaling early in the evolution of nervous systems and has become the most ubiquitous neurotransmitter, as evidenced by its inclusion in nearly 95% of cortical synapses (Kasthuri et al., 2015; Moroz et al., 2014; T. J. Ryan & Grant, 2009; Schousboe, 1981). Indeed, relatively recent adaptations such as the expression of novel, functionally distinct NMDAR subunits contributed to enhanced signaling complexity in synapses which expanded the amount of information that could be encoded by glutamate (Teng et al., 2010). Beyond this, the human brain has been further equipped with glutamate receptors and release mechanisms (Lewerenz et al., 2013; Malarkey & Parpura, 2008), expressed by non-neuronal cells and in non-synaptic domains (Chiu, DeSalle, Lam, Meisel, & Coruzzi, 1999; Papouin et al., 2017; Tikhonov & Magazanik, 2009). A major question is whether or not these non-synaptic, non-neuronal adaptations are relevant to the expansion of signaling complexity within neural networks and were essential to the enhanced cognitive ability of higher vertebrates.

The acquisition of novel genes encoding astrocytic glutamate-release mechanisms that signal within non-synaptic domains may have been key in expanding the processing power of neural circuits and consequently, cognitive abilities. In support, a human cortical astrocyte contacts millions of synaptic units, which is considerably more than neurons and allows them to communicate with and regulate entire neural networks (DeFelipe, Alonso-Nanclares, & Arellano, 2002; Oberheim et al., 2009; Oberheim et al., 2006). Indeed, computational models reveal that astrocyte-neuron signaling significantly improves the

performance of neural networks, especially when the size of the network or the complexity of the information is maximized (Alvarellos-Gonzalez et al., 2012; Porto-Pazos et al., 2011). Additionally, biological studies demonstrate that astrocytes exert complex regulation over neural networks, which can include the gating of specific circuits or increased network synchronization (H. S. Lee et al., 2014; Martin et al., 2015; Poskanzer & Yuste, 2016; Sardinha et al., 2017). While there are a variety of mechanisms that release glutamate from astrocytes (Malarkey & Parpura, 2008) there is a critical gap in our understanding of their individual and collective roles in neural network regulation and cognition. Consequently, there has only been a recent appreciation for the possibility that astrocytes are active players in cognition which is striking considering the degree of influence they exert over neural networks (Santello et al., 2019).

In the studies described in this chapter, we investigated the role of the cystine-glutamate antiporter system x_c^- (Sxc) in the regulation of neural circuits and cognition. Sxc releases intracellular glutamate in exchange for extracellular cystine (Baker et al., 2002; Warr, Takahashi, & Attwell, 1999). It is a heterodimer of the subunits xCT and 4F2hc, which are encoded by the genes *Slc7a11* and *Slc3A2*, respectively. Importantly, the xCT subunit serves as the catalytic subunit whereas the 4F2hc guides and anchors it in the membrane (de la Ballina et al., 2016; Sato, Tamba, Ishii, & Bannai, 1999; Shih & Murphy, 2001). Intriguingly, the observed expression patterns of *Slc7a11* establish Sxc as an evolutionarily-new glutamate-release mechanism expressed by astrocytes (Lewerenz et al., 2013; Ottestad-Hansen et al., 2018) and is present in brain regions involved in

cognitive processing such as the hippocampus, amygdala, prefrontal cortex, and nucleus accumbens (Baker, McFarland, Lake, Shen, Tang, et al., 2003; Lutgen et al., 2014).

In the nucleus accumbens core (NAcc), Sxc activity is negatively impacted by self-administration of drugs of abuse which has made it a focus for therapeutic intervention in substance abuse patients (Kalivas, 2009). In support, Sxc activity is reduced following self-administration of cocaine in rats resulting in reduced extracellular glutamate in the NAcc. This reduction is thought to produce drug seeking through a mGluR2 dependent mechanism whereby a reduction in its activity produces a disinhibition of glutamate release from cortical afferents; enhancing excitatory drive on NAcc efferents and consequently promoting drug seeking (Baker et al., 2002). Intriguingly, restoring extracellular glutamate levels using drugs that may target Sxc such as n-acetylcysteine reduces drug seeking during drug primed reinstatement tests (Baker, McFarland, Lake, Shen, Tang, et al., 2003). The use of n-acetylcysteine has also shown efficacy in clinical models of cocaine addiction, where a four-day treatment regimen attenuated cocaine craving following an I.V. infusion (Amen et al., 2011). Furthermore, n-acetylcysteine has a similar efficacy in preclinical and clinical studies of heroin (Zhou & Kalivas, 2008) and nicotine (Schmaal et al., 2011) addiction as well as other compulsive disorders such as binge eating (Hurley et al., 2016) and Prader-Willi syndrome (J. L. Miller & Angulo, 2014). These studies lend support to Sxc having a significant impact on neuronal signaling and behavioral control and is a promising therapeutic target.

Given the novelty of Sxc expression in animal species, its ability to regulate synaptic glutamate, and its potential to influence cognition, we sought to further elucidate the biological significance of its expression in a mammalian system. To do this, we developed a novel rat strain that lacks functional Sxc (Figure 2-1B, MSxc rat). With this tool, we tested the hypothesis that global removal of Sxc in a rat would sufficiently disrupt neural networks and cognition in a manner that produces maladaptive behavioral phenotypes. Furthermore, the MSxc rat will help resolve whether astrocyte to neuron signaling was a key event in achieving the signaling complexity needed for executive function and cognition in mammals.

MATERIALS AND METHODS

Chemicals: Unless noted, common chemicals and reagents were obtained from Sigma Aldrich.

Animal care and usage: Male Sprague Dawley rats were used for these experiments. Housing conditions and experimental protocols were approved by Institutional Animal Care and Use Committees at the Medical College of Wisconsin or Marquette University, and were carried out according to the US National Institutes of Health guidelines.

SLAC Analysis: mRNA protein coding sequences from the *SLC7A11* gene from 115 organisms were retrieved from Genbank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2009). Partial and isoform sequences were left out of this study. Sequences were aligned by codon using MUSCLE (Edgar, 2004) in the MEGA6 software package(Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and

manually reviewed for accuracy. The aligned sequences were run through MEGA6 model selection software using a neighbor-joining tree and a maximum-likelihood statistical method. Gaps were treated by partial deletion and were not used for computing tree branch lengths. The K2+G (gamma distributed) model was utilized to construct a phylogenetic tree. Pairwise distances were estimated with uniform rates and partial deletion of gaps. Bootstrap values were generated from 100 replications. A cutoff tree was computed with a minimum bootstrap value of 70. Sequence motifs for specific consensus phosphorylation sites were searched within the alignment and conservational data was manually extracted based on the existence or absence of the site. Determination of sites under positive or negative selection was carried out using the Datamonkey web server (Delport, Poon, Frost, & Kosakovsky Pond, 2010). SLAC, branch-based SLAC, FEL, and an integrative selection analysis (Kosakovsky Pond & Frost, 2005) were carried out using a neighbor joining tree at a 0.05 significance level.

Creation of GFAP:Lck-eGFP transgenic rats: The Sleeping Beauty (SB) transposon transgenesis method (Geurts et al., 2010) was used to produce transgenic rats on the Sprague Dawley (Crl:SD) background. Briefly, a GFAP promoter-driven fusion between the plasma membrane targeting myristolation domain of the mouse LCK protein fused to eGFP(Benediktsson, Schachtele, Green, & Dailey, 2005) was synthesized (GeneArt) and cloned into a SB transposon vector. The transposon plasmid was co-injected with a source of

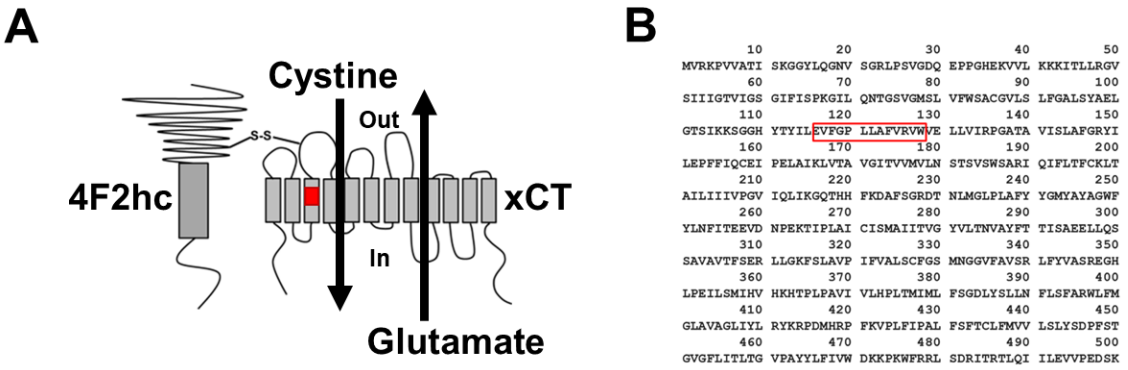


Figure 2-1: Quaternary and primary structure of Sxc and xCT. A, The quaternary structure of Sxc is comprised of the heavy chain subunit (4F2hc a.k.a. CD98) and the transporter subunit xCT which contains 12 transmembrane domains. **B,** The primary structure of the protein coding sequence of xCT. Area highlighted in red defines the target for the mutation that created the xCT mutant (MSxc) rat which corresponds to the third transmembrane domain seen in **A**.

SB100X transposase mRNA into the pronucleus of fertilized Crl:SD embryos. A breeding colony was established harboring a single copy of the transgene inserted on rat chromosome 13 enabling robust eGFP expression. To confirm expression *in situ*, a rat was deeply anesthetized and transcardially perfused with 4% paraformaldehyde. The brain was extracted and sliced at 100 µm and GFP signal was visualized using standard fluorescence microscopy.

Creation of MSxc rats: Zinc-finger nucleases (ZFNs) were designed targeting the second exon sequence (TGCTAGCTTTTGTTCgagtcTGGGTGGAACTGC TG) to produce small deletions of a limited number of base pairs in the *Slc7a11* gene, where capital letters represent binding sites for the individual ZFN monomers, on opposite strands (Figure 2-1). ZFNs were injected into the pronucleus of Sprague Dawley (Crl:SD) rat embryos by pronuclear microinjection of in vitro-transcribed encoding messenger RNAs and the resulting offspring were screened for mutations using a Cel-1 assay and validated by Sanger

sequencing as previously described(Geurts et al., 2010) and resulting in single-step, whole-animal disruption of *Slc7a11* (MSxc rats). Deletion of 39 consecutive base pairs (GAGGTCTTTGGTCCCTTGCTAGCTTTTGTTCGAGTCTGG) of exon 2 was confirmed by Sanger sequencing.

Tissue Dissociation for FACS: NAc tissue was dissected from male GFAP-lck-eGFP or WT Sprague Dawley rats and placed into ice-cold Hanks´ Balanced Salt Solution without Ca^{2+} or Mg^{2+}. Tissue was mechanically and enzymatically dissociated to obtain single-cell suspension using Neural Tissue Dissociation Kit - P (Miltenyi Biotec). 1% BSA, 1mM EDTA and 12.5 U/ml DNase I were added to reduce cell clustering. Cells were incubated with 3 µM Calcein Violet 450 AM Viability Dye (eBioscience) on ice for 10 minutes to stain live cells. An aliquot from each cell suspension was used to determine relative gene expression in sorted and unsorted (total) cells.

Flow Cytometry: FACS analyses were performed using FACSDiva software 6.1.3 (BD Biosciences). Control samples (tissue obtained from rats lacking the GFAP:Lck-eGFP transgene) were analyzed first to identify the range of endogenous or background fluorescence intensity. In addition, the gating parameters of the flow cytometer were set to isolate live, eGFP+ cells from cellular debris, and cell doublets. Note, the eGFP- samples contain all tissue excluded from the eGFP+ sample and would be expected to include eGFP- cells, cell doublets, fragmented cells, and potentially eGFP+ cells that were not excluded due to the use of strict gating parameters or incomplete cellular disassociation.

RNA Extraction and Amplification of FACS samples: Total RNA from sorted cells was isolated using PicoPure RNA Isolation Kit (Thermo Fisher Scientific). On column DNase treatment was applied to all samples with the RNase-Free DNase Set (Qiagen). RNA quantity and quality were assessed on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Chips. Only samples with RNA quality of RIN > 7.0 were used. Resulting RNA was amplified and reverse transcribed using the cDNA synthesis from cell lysates kit (Lucigen).

RT-PCR/qPCR: Total RNA was extracted from NAcc tissue samples using Trizol reagent and was subsequently treated with DNAse (Life Technologies) to remove genomic DNA contamination. RNA purity and quantity were assessed using a Nano Vue Plus spectrophotometer (GE Life Sciences). RNA (1 μg) from each sample was reverse transcribed for PCR (Promega). PCR was conducted using GoTaq DNA polymerase (Promega). For analysis of FACS samples, qPCR was conducted using SYBR green chemistry (Quantabio) in a 48-well StepOne real-time PCR system (Applied Biosystems). Primer sequences were as follows: Slc7a11 (xCT) forward- 5’ AGG GCA TAC TCC AGA ACA CG 3’; Slc7a11 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’. 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’.

Western Blotting: NAcc tissue samples obtained from adult WT and MSxc rats (i.e., > 90 days old) underwent mechanical homogenization in a sucrose-based

buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.4) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration for each whole cell lysate sample was determined via the bicinchoninic acid assay (BCA). Protein (10 μg) was resolved in a 7.5% polyacrylamide gel and wet transferred to Immobilon PVDF membranes (EMD Millipore). Membranes were immersed for one hour in blocking buffer (LiCor) prior to overnight application at +4°C with a primary antibody: rabbit anti-mGluR2/3 (Upstate, 1:3000) rabbit anti-EAAT2 (1:3000) rabbit anti-pT840 GluA1 (Abcam, 1:3,333) rabbit anti-pS845 (Abcam, 1:1000) mouse anti-GluA1 (EMD Millipore, 1:1000) chicken anti-GAPDH (EMD Millipore, 1:10,000) rabbit anti-xCT (generated by Dr. Rothstein, 1:500). Secondary antibodies (LiCOR) goat anti-rabbit IR 680 DYE (1:15000) donkey anti-mouse IR 800 DYE (1:20,000) and donkey anti-chicken IR 800 DYE (1:20,000) were applied for 1 hour at room temperature prior to imaging (Odyssey Fc Imaging System, Licor). Band intensity was quantified using LiCOR Image Studio software and the resulting data are presented as the target protein band intensity divided by the reference protein band intensity.

Cell Culture: Astrocyte cultures were generated from postnatal day-3 rat pups. The striatum was dissected and dissociated using 0.25% trypsin EDTA (Gibco) and cultured in 75 cm^2 flasks in a humidified incubator at 37°C under 95% O_2 5% CO_2 in Eagles minimum essential medium (Gibco) supplemented with 5% fetal bovine serum/5% horse serum (Atlanta Biologicals), Glutamax (Gibco), and antibiotics/antimycotics (Gibco). To remove debris and non-astrocytic glia, flasks were agitated, and the resulting mono-cell layer was resuspended with 0.25%

trypsin EDTA. Cells were counted by hand via a cytometer and seeded in 24-well plates coated with poly-D-lysine and laminin at a density of 200,000 cells per well.

^{14}C-L-Cystine and ^{3}H-D-Aspartate uptake assays: These assays were used to determine Sxc and EAAT function, respectively. NAcc tissue punches or astrocyte cultures obtained from adult WT and MSxc rat were incubated for 20 minutes in artificial cerebral spinal fluid (aCSF) and maintained at 37^{o}C under 95% O_2 5% CO_2 conditions. For D-aspartate uptake, NAcc tissue or cultured cells were then incubated for 30 minutes in 1 μM ^{3}H-D-aspartate dissolved in aCSF. Likewise, for ^{14}C-L-Cystine (1 μM), tissue was incubated for 30 minutes. For each study, tissue punches or cells were then dissolved in 300 μl of 1% sodium dodecyl sulfate (SDS). A 200 μl aliquot was used for scintillation counting (Beckman 6500) and a 25 μl aliquot was used for protein determination using the BCA assay.

Cystine-evoked glutamate release assay: In order to measure Sxc function, striatal astrocytes (DIV14) were incubated for 30 minutes at 37^{o}C in Na^{+}-free buffer containing the following: 116 mM choline chloride, 13.4 mM MgSO4, 1.68 mM KH_2PO_4, 2.34 mM $CaCl_2$, 5.49 mM dextrose, 11.9 mM HEPES, 0.2% choline bicarbonate, titrated to pH: 7.4 with CeOH. This buffer was used to prevent Na^{+}-dependent uptake of glutamate. Increasing concentrations of L-cystine 0, 12.5, 25, 50, 100, 200 μM were applied to drive cystine-glutamate exchange by Sxc. Media samples (100 μl) were collected for subsequent glutamate analysis using

high performance liquid chromatography (HPLC). Cells were then dissolved in 0.5% SDS and total protein for each well was quantified using the BCA method.

Glutamate HPLC: The concentration of glutamate was quantified by comparing peak areas from samples and external standards using HPLC coupled to fluorescence detection. A 10 μl sample underwent pre-column derivatization with orthophthaladldehdye (OPA) in the presence of 2-mercaptoethanol using a Shimadzu LC10AD VP autosampler. Chromatographic separation was achieved using a Kinetex XB C-18 (50 x 4.6 mm, 2.6 μm; Phenomenex) and a mobile phase consisting of 100 mM Na_2HPO_4, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% acetonitrile at a pH of 6.04. Glutamate was detected using a Shimadzu 10RF-AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively. Glutamate content for each sample was normalized to total protein in the respective well and depicted as a net change from baseline. The data was fit using the Michaelis-Menten equation to determine Km and Vmax.

Rhodamine tracer injection: In order to label NAcc efferent pathways, adult rats were anesthetized with ketamine/xylazine and underwent stereotaxic surgery. Guide cannula (Plastics One) were implanted into the brain targeting the SN (0°, -5.0 mm anterior-posterior, +2.1 mm medial-lateral, -6.1 mm dorsal-ventral) or VP (0°, -0.6 mm anterior-posterior, +2.8 mm medial-lateral, -6.3 mm dorsal-ventral). Micro-injectors (Plastics One) were inserted into guide cannula and extended at least +2.0 mm from the distal aspect of the guide cannula for placement into the target structure. Rhodamine fluorescent latex microspheres (300 nl; Lumafluor)

were injected into the target region. Animals recovered for at least 2 weeks prior to use in experiments.

Brain slice preparation and electrophysiology: Adult rats were anesthetized by isoflurane inhalation and perfused through the aorta with a cold sucrose-based solution (4–6 °C) containing (in mM): 78 NaCl, 68 sucrose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$ and 25 glucose. The 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). Slices were initially incubated in the above sucrose-based solution at room temperature (30-40 min) and were then transferred and stabilized (30 min) in an aCSF solution containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. All solutions were saturated with 95% O_2 and 5% CO_2. Whole-cell recordings were made using patch clamp amplifiers (Multiclamp 700B) under infrared-differential interference contrast optics (Nikon Eclipse FN1 and Olympus BX51WI) and a 40x water immersion lens. Data acquisition and analysis were performed using DigiData 1440A and 1550B digitizers and analysis software pClamp 10 (Molecular Devices). Signals were filtered at 2 kHz and sampled at 10 kHz. Whole-cell patch-clamp recordings were made from rhodamine bead-labelled medium spiny neurons (MSNs) in NAcc slices. MSNs were also identified visually by their medium-sized, spindle-like cell bodies, large initial axon segment, and electrophysiological characteristics as described in our previous studies (Liu et al., 2013; Wang et al., 2010). Glass pipettes (4-6 MΩ) were filled

with one of the following internal solution (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 1 EGTA-Na, 2 Mg-ATP, 0.3 Na-GTP, and 10 Na_2-phosphocreatine at pH 7.2 (with KOH). Neurons were voltage-clamped at −70 mV. A bipolar tungsten-stimulating electrode was placed at the prelimbic cortex–NAcc border to stimulate afferent cortical fibers. The $GABA_A$ receptor blocker picrotoxin (100 μM) was freshly prepared and dissolved in aCSF using sonication (~10 min). For the recordings of paired-pulse ratio, two consecutive stimuli with 20-400 ms intervals were applied. For the recordings of miniature excitatory postsynaptic currents (mEPSCs), the Na^+ channel blocker tetrodotoxin (TTX, 0.5 μM, Tocris) was added to the aCSF solution to block action potentials.

Physiology Telemetry: Adult rats were implanted intraperitoneally with telemetry probes (Mini-Mitter Inc.) to remotely record core body temperature and locomotor activity while in their home cage. Temperature and activity data were collected every 5 minutes and averaged in 1-hour bins.

Open Field: Adult rats were placed into an open-field chamber (150 x 100 x 40 cm) and oriented toward a wall to begin the 15-minute session. The amount of time animals spent in the center zone (defined as 15 cm from each wall) and the total distance travelled was recorded using video-tracking software (EthoVision).

Novel Object Recognition: During the first two days, rats underwent five-minute habituation sessions, which involved placing the rats in a 50x25cm bedding-free chamber outfitted with a camera. On the third day, rats were placed into the maze for five minutes to become familiarized with two identical objects that were placed in adjacent corners of the chamber. One hour later, rats were placed into

the maze for five minutes, which had one “familiar” object and one novel object. The time spent interacting (looking/sniffing/climbing) with each object was recorded during each session on the third day. The placement and identity of each object were randomized to control for object and spatial preference.

Pavlovian Conditioned Approach (PCA) and Omission testing: Adult WT and MSxc rats were subjected to an PCA (also known as autoshaping) paradigm using touch screen operant chambers (Campden Instruments) (Horner et al., 2013). Briefly, rats were food deprived to approximately 90% of their *ad libitum* mass and handled daily for a week prior to training. On the first day of training, animals were habituated to the chambers for 20 minutes with 20 chocolate flavored sucrose pellets placed in the reward delivery tray. If the subject consumed all of the pellets, they progressed to the second phase of habituation which entailed a 30-minute training session whereby following a variable intertrial interval (0-30s) a reward is delivered in the tray concurrent with a tray light and a 1 second 3 KHz sound. If 40 trials are completed, i.e. 40 rewards consumed, the subject progressed to PCA training. PCA training entailed five days of 20 presentations of CS+ and CS- for a total of 40 total trials. Cues were presented when both a variable intertrial interval had passed and the infrared beam in the back of the chamber was broken. Cues were presented for 10s and if CS+ was presented, cessation was immediately followed by reward delivery with concurrent tray light and a 1 second 3 KHz sound. Omission testing was the same, however if the rat approached CS+ when it was presented, a reward would not be delivered.

Fear Blocking and Extinction: Adult rats were trained using a within-subjects Kamin fear blocking design (Furlong, Cole, Hamlin, & McNally, 2010). This design consisted of four stages across 5 consecutive days: stimulus pre-exposure (Stage 0), fear conditioning (Stage 1; 2 days), blocking (Stage 2), and testing (Stage 3). Two 30-second auditory cues and two 30-second visual cues were used in this experiment: CSA, constant cue-light illumination; CSB, 72-dB white noise; CSC, 1-Hz flashing cue-light; CSD, 72-dB 6-kHz tone. Chambers used for training (Stages 0-2) and testing (Stage 3) differed in texture and odor. In each session, rats were acclimated to the chamber for 120 sec before stimulus presentation (Baseline period). The inter-trial interval was 60 sec in Stages 0 and 3 and 240 ± 20 sec in Stages 1 and 2. During Stage 0, rats were presented with each of the four CSs twice in a pseudorandom order. During Stage 1, rats received four presentations of Cue-A co-terminating with a 1-sec foot-shock unconditional stimulus (UCS; 0.5 mA) on each of two days. During Stage 2, rats received alternating presentations (2 each) of compound cues AB and CD, co-terminating with the UCS. During Stage 3, rats were briefly returned to the training chamber for 10 minutes in the absence of stimuli to assess contextual fear. Two hours later, rats were tested in a novel chamber for fear to Cue-B and Cue-D in the absence of foot shock (Stage 3) with each stimulus being presented four times. At least seven days following the completion of the fear blocking experiment, all rats received an extinction session in the testing chamber. This session consisted of 40 unreinforced presentations of Cue-A (30 sec) with an inter-trial interval of 60s. The following day, rats were tested for their extinction

memory; they were again placed in the extinction chamber and received 8 unreinforced CSAs. Freezing was defined as the cessation of all movement except that needed for respiration and was used as the measure of conditional fear during all training and testing sessions(Fanselow & Bolles, 1979). Freezing was scored automatically using FreezeScan 2.0 (CleverSys).

Attentional Set Shifting: Adult rats were food-restricted to 90% of their ad libitum body mass for one week. Next, rats were habituated to a four-arm cross maze (60 x 20 x 12 cm). For each of the three test sessions, rats were run in blocks of 12 trials. For each trial, only three of the four arms were made accessible by blocking the fourth arm, thereby creating a T shaped-maze. In this configuration, one arm was used as a starting chamber; the arms to the left and right of the starting location were used as choice arms. Each of the choice arms had a small divider at the distal end to prevent subjects from visually identifying which choice arm contained a sucrose pellet. On each of the three test sessions, a visual cue (a laminated paper with black and white stripes) was placed in one of the choice arms. Following the completion of 12 trials, the configuration of the maze rotated such that the arms representing the starting and choice arms could be adjusted, thereby minimizing the utility of spatial cues outside the maze. Testing continued until the subjects successfully identified the baited arm in ten consecutive sessions. On the first daily test session, rats needed to acquire a simple visual discrimination to learn that the placement of the sucrose pellet was indicated by the placement of the visual cue. On the second daily test session, rats were required to utilize an extradimensional shift since the location of the

sucrose pellet was indicated by a directional cue (e.g., always turn right or always turn left) rather than a visual cue (which now indicated the location of the sucrose pellet at chance levels). On the third day, rats were required to display reversal learning since the solution involved a directional cue that was opposite to that of day 2. Perseverative errors were calculated on days 2 and 3 and represented errors due to rats' applying the prior day's strategy. Regressive errors represented mistakes occurring after identification of the trial strategy (i.e., at least 75% correct within a block of 8 trials) but prior to achieving the trial criterion (10 consecutive test trials).

Cocaine Self-Administration and Reinstatement: Adult rats were trained to self-administer cocaine (0.5 mg/kg/inf, IV; 2 hr/day) or saline under a fixed-ratio one schedule of reinforcement for 12 daily sessions (Madayag et al., 2010). Rats then underwent a seven-day abstinence period, followed by extinction training sessions, which were identical to the self-administration sessions except lever presses resulted in saline infusions. Once rats met the extinction criterion of fewer than 15 presses/day, reinstatement testing was conducted. Reinstatement testing involved pretreatment with a low dose of cocaine (3 mg/kg, IP) ten minutes prior to testing and recording lever pressing for 2 hours.

Statistical Analysis: Investigators were blinded to genotype for all behavioral procedures. All data are presented as means $\pm$ standard error of the mean. Analyses of data sets comprised of only 2 groups were analyzed using two-tailed Student's t-test or the Kolmogorov-Smirnov test (K-S test) for nonparametric analyses. Analyses of data sets comprised of three or more groups were

analyzed using analysis of variance with Tukey post hoc test. To calculate PCA scores during PCA training the following equation was used:

Analysis	Variable	Equation
Response Bias (C)	**CS+ Approach During CS+ (A)** **Tray Entries During CS+ (B)**	**C = (A-B)-(A+B)**
Probability Difference (F)	**CS+ Approach Trials (D)** **Tray Entry Trials (E)**	**F = (D/20)-(E/20)**
Latency Score (I)	**Averaged Tray Entry Latency (G)** **Averaged CS+ Approach Latency (H)**	**I = (G-H)/10**
		PCA Score = (C+F+I) / 3

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). The paired-pulse ratio (PPR) was calculated by dividing the mean amplitude of the second EPSCs by that of the first EPSCs. Results were deemed significant at $p<0.05$.

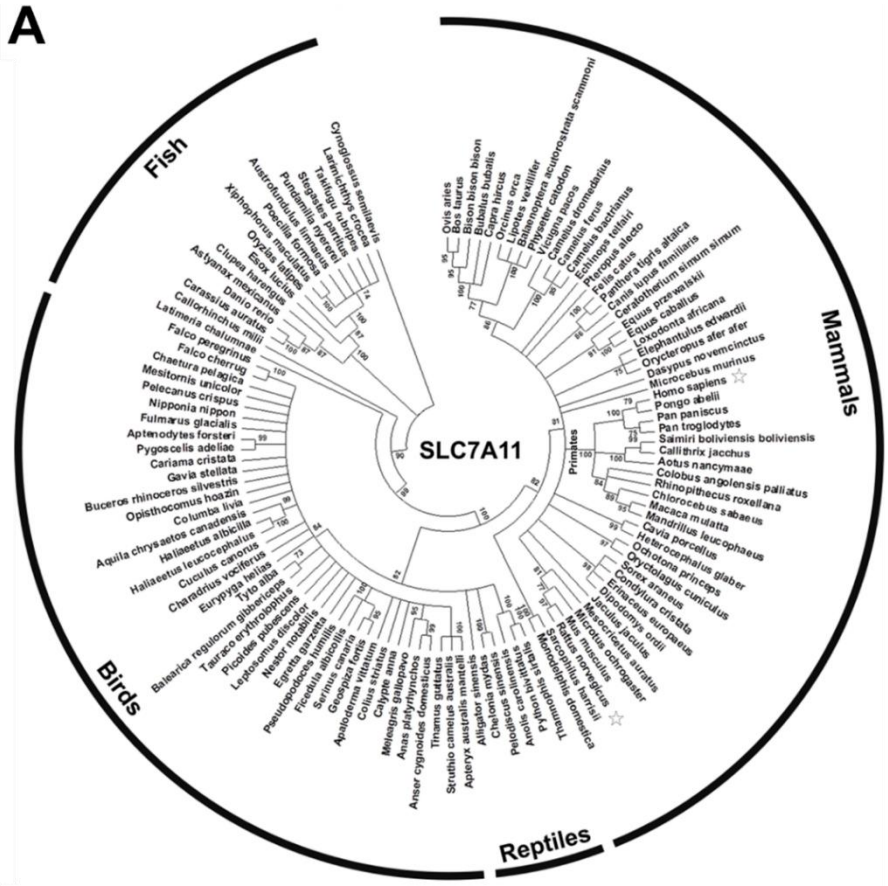
RESULTS

Intriguingly, the xCT subunit of Sxc is a relatively recent adaptation for animal species (Lewerenz et al., 2013). Hence, analyzing the degree of spontaneous mutations within the xCT sequence over the course of the evolution of vertebrate species we could infer its functional importance to these organisms. To determine this, a phylogenetic tree was constructed to help infer ancestral divergence points based on the conservation of the sequence. The tree reveals a pattern of divergence consistent with canonical vertebrate species trees whereby the traditional classes (e.g. birds, reptiles, and mammals) form separate clades (Figure 2-2A). Intriguingly, primates form their own clade which is suggestive of

significant changes to the sequence giving rise to a unique xCT sequence relative to other mammals.

One possibility is that the speciation event giving rise to primate xCT was driven by the acquisition of novel kinase regulatory sites which could change its function. Thus, the sequence was scanned for consensus phosphorylation sites which revealed three candidate regions (Figure 2-2B). First, we detected serine residue 26 (S26, CaMKII/MSK1 site) and found it to be present in 100% of the primates analyzed but only in 45% of non-primate mammals. Next, S481 (CaMKII/MSK1 site) is only present in 79% non-primate mammals and 100% of primates. Last, S185 (GSK3-β site) was present in 92% of vertebrate species. This suggests that novel regulatory sites on the xCT sequence were acquired during vertebrate evolution and may have given rise to speciation events or provided an adaptive advantage to the species that acquired them. In the case of humans and other animals, this could include more complex signaling leading to more complex cognition or behavior.

Considering the acquisition of these regulatory sites, we next asked whether there was evidence of the xCT sequence evolving under the influence of positive or negative selective pressure. Positive selective pressure favors rapid genetic changes that provide an adaptive advantage whereas negative selective pressure works to resist sequence changes by purifying changes from a



Class	Total Analyzed	CaMKII/MSK1 R-L-P-S[26]	CaMKII/MSK1 R-I-M-S[481]	GSK-3 S-M-S-V-S[185]
Cartilaginous Fishes	1	0 (0%)	0 (0%)	1 (100%)
Fringe-finned Fishes	1	0 (0%)	0 (0%)	1 (100%)
Ray-finned Fishes	14	0 (0%)	0 (0%)	5 (36%)
Reptiles	6	3 (50%)	0 (0%)	6 (100%)
Birds	39	31 (79%)	0 (0%)	39 (100%)
Mammals	42	19 (45%)	33 (79%)	42 (100%)
Primates	12	12 (100%)	12 (100%)	12 (100%)
TOTAL	***115***	***65 (57%)***	***45 (39%)***	***106 (92%)***

Figure 2-2: Phylogenetic analysis of vertebrate xCT. A, Phylogenetic tree illustrating the xCT protein coding sequence relationship across 115 vertebrate species. Major ancestral divergence points are evidenced by branch points (all greater than 70 bootstrap). **B**, The percent of species within vertebrate classes that express designated xCT residues targeted by their respective kinase; in order from left to right S26, S481, S185. **C,** Data depicts the degree of non-synonymous (dNS) to synonymous mutations (dS; dN-dS) across the xCT sequence. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; MSK1, mitogen and stress activated kinase 1; GSK3-β, glycogen synthase kinase-3 beta.

population through a reduction in fitness i.e. acquisition of maladaptive traits (Eric J. Vallender & Lahn, 2004). We hypothesized that the regulatory sites may have

given the species that acquired them an adaptive advantage i.e. were positively selected for. To test, a single-likelihood ancestor count (SLAC) analysis was used to ascertain the degree of selective pressures driving these sequence changes across vertebrate evolution (Kosakovsky Pond & Frost, 2005). This analysis revealed that 395/564 (70%) of codons analyzed displayed a significantly higher degree of nucleotide mutations that did not alter the amino acid sequence (degree of synonymous mutations; dS) than mutations that did alter the amino acid sequence (degree of non-synonymous mutations; dNS; $p<0.05$; Figure 2-2C). There was no evidence for positive selective pressure in the xCT sequence (0/564, $p>0.05$). The regulatory sites S185 and S481 were under significant negative selective pressure ($p<0.05$) whereas S26 trended toward negative selective pressure ($p=0.08$). These findings suggest that S26, S185, and S481 did not provide an adaptive advantage to species that gained them, rather their acquisition was rapidly stabilized and resisted further change. Thus, xCT largely evolved under negative selection whereby changes to the sequence would likely produce an organism with maladaptive traits. Collectively, these data suggest that the phylogenetic signature of xCT indicates it was an essential protein to vertebrate species.

Having established the phylogenetic importance of xCT to vertebrates, we next sought to further strengthen our understanding of the cellular expression profile of xCT. Models of glutamate signaling depict Sxc as an astrocytic mechanism, but the *in vivo* support for this is limited (Ottestad-Hansen et al., 2018). The GFAP:Lck-eGFP rat was developed to assist in isolating fluorescently

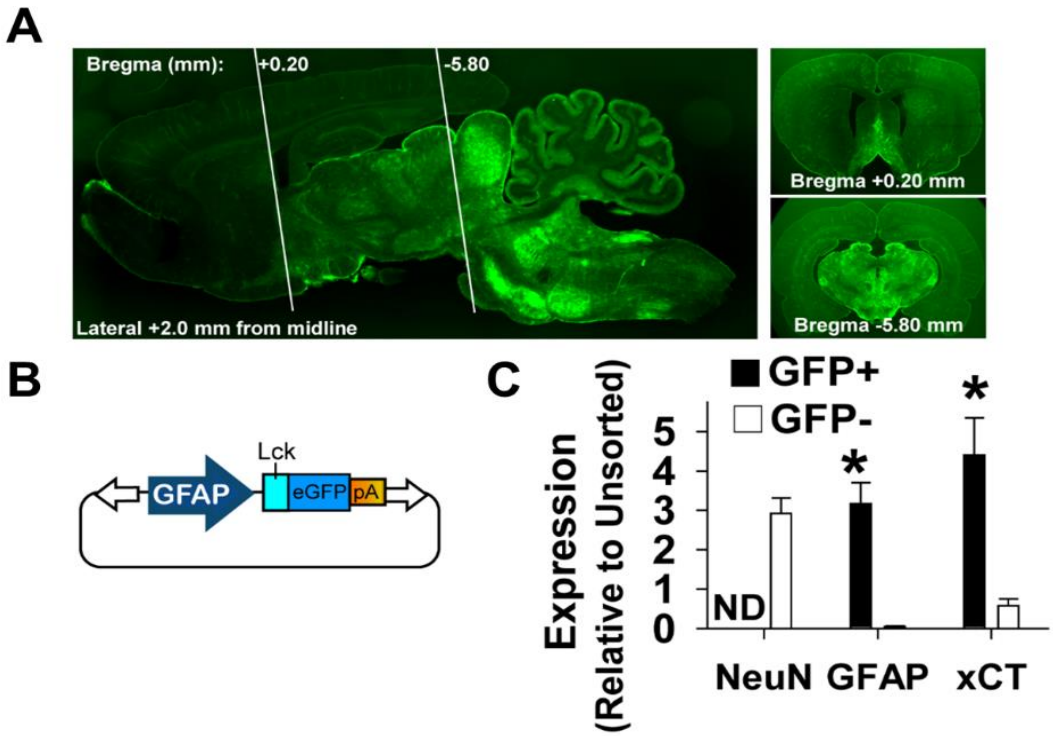


Figure 2-3. xCT is expressed by GFAP positive cells in the NAcc.
A, Fluorescence photomicrograph of GFP expression in the rat brain. **B,** The transgene used to fluorescently label-astrocytes in GFAP:Lck-eGFP rats. **C,** Levels of mRNA for the neuronal control gene NeuN, the astrocytic control gene GFAP, and xCT in FACS-sorted GFP+ and GFP- cells. *p<0.05, t-test. Special thanks to Dr. Alex Savtchouk for A.

labeled astrocytes from adult NAcc tissue via FACS in order to quantify xCT expression in these cells (Figure 2-3B). The NAcc was chosen for our molecular and cellular experiments since this subcortical structure integrates inputs from throughout the brain and is consequently an essential nucleus for cognitive processing (Floresco, Ghods-Sharifi, Vexelman, & Magyar, 2006; Svensson & Ahlenius, 1983). Furthermore, glutamate signaling in this structure relies on Sxc activity (Baker, McFarland, Lake, Shen, Toda, et al., 2003; Baker et al., 2002; M. M. Moran, McFarland, Melendez, Kalivas, & Seamans, 2005). Gross fluorescence microscopy demonstrates eGFP expression throughout the brain (Figure 2-3A). QPCR revealed a lack of NeuN expression in GFP+ cells.

Additionally, GFAP and xCT expression was significantly higher in GFP+ cells relative to GFP- (Figure 2-3C; n=4/genotype: GFAP, t_6=5.79, p<0.05; xCT, t_6=4.01, p<0.05). These data demonstrate that GFAP positive cells, astrocytes, are the predominant cell type in the NAcc that expresses xCT.

Given the phylogenetic significance and the expression of xCT in astrocytes, we next sought to determine the physiological and behavioral impact of mutating xCT in a mammalian system. We hypothesized that this would produce an animal with maladaptive traits. The MSxc rat was developed using Zinc-finger nuclease technology which yielded rats that lacked xCT protein and mRNA (Figure 2-4A-B). Next, an *in vitro* cystine-evoked glutamate release assay was conducted to test if Sxc was functional. This revealed that cystine uptake significantly increased extracellular glutamate in WT but not MSxc astrocytes (Figure 2-4C; cystine, $F_{5,79}$=1.736, p>0.05; genotype, $F_{1,79}$ =47.963, p<0.05; genotype x cystine, $F_{5,79}$=2.67, p<0.05). Genotypic effects were present within 25, 50, 100, and 200 μM cystine (Tukey, p<0.05). Additionally, ex-vivo slices of the NAcc from WT and MSxc rats were incubated in ^{14}C-L-cystine in the presence or absence of the Sxc inhibitor sulfasalazine (SSZ) to determine if Sxc-dependent cystine uptake was ablated. We report effects of genotype, SSZ, and an interaction between these variables (Figure 2-4D; n=10-12 per genotype/treatment; genotype, $F_{1,42}$=54.392, p<0.05; SSZ, $F_{1,42}$=53.221, p<0.05; genotype x SSZ, $F_{1,42}$=49.953, p<0.05). There was a significant effect of

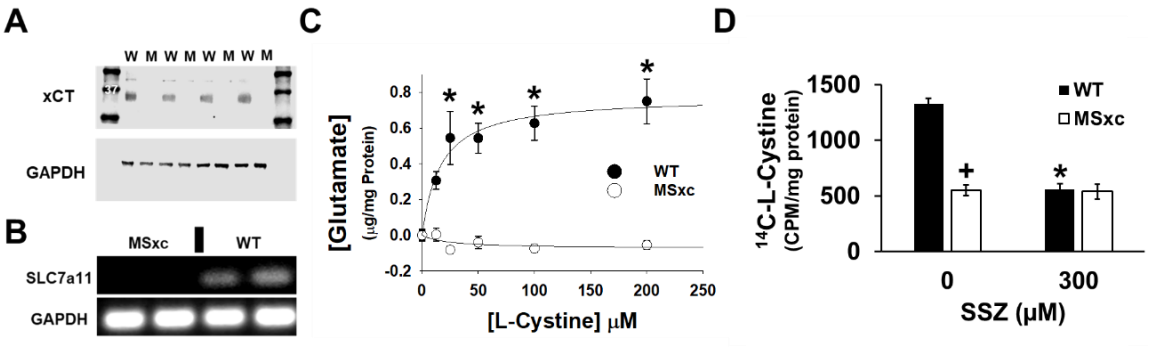


Figure 2-4: Validation of a Sxc-deficient rat model. A-B, xCT protein and mRNA expression, respectively, in NAcc tissue samples obtained from WT and MSxc rats. **C**, Cystine-evoked glutamate release was in cultured striatal astrocytes generated from PD3 WT and MSxc tissue. **D,** Radiolabeled cystine uptake in NAcc ex-vivo slices from WT and MSxc rats in the presence or absence of the xCT inhibitor sulfasalazine (SSZ). Two-way ANOVA, Tukey test; **C,** * effect of genotype within given cystine, $p<0.05$; **D,** * effect of treatment within genotype; + effect of genotype within treatment, $p<0.05$. Special thanks to Dr. Jeffrey Rothstein for the xCT antibody used in A.

genotype within vehicle and an effect of treatment within WT (Tukey, $p<0.05$). These data demonstrate that Sxc is functionally knocked out in MSxc rats.

To investigate the physiological and behavioral impact of loss of Sxc function we first utilized paradigms that would test for genotypic differences in physiological telemetry, development, exploratory behavior, and simple associative learning which would indicate the presence of maladaptive traits. We report a lack of a genotypic effect on postnatal growth rate (Figure 2-5A; n=25-28/genotype; effect of genotype $F_{1,51}=0.33$, $p>0.05$; week: $F_{4,204}=22.18$, $p<0.001$; genotype x time: $F_{4,204}=4.20$, $p<0.001$; $p> 0.05$ when comparing genotype at each week), diurnal core body temperature (Figure 2-5B; n=7-8/genotype; genotype x time $F_{120,1560}=0.952$, $p>0.05$; genotype $F_{1,13}=0.113$, $p>0.05$; time $F_{120,1560}=51.133$, $p<0.001$), and home cage locomotor activity (Figure 2-5C; n=12/genotype; genotype x day $F_{7,91}=0.458$, $p>0.05$; genotype $F_{1,13}=0.331$,

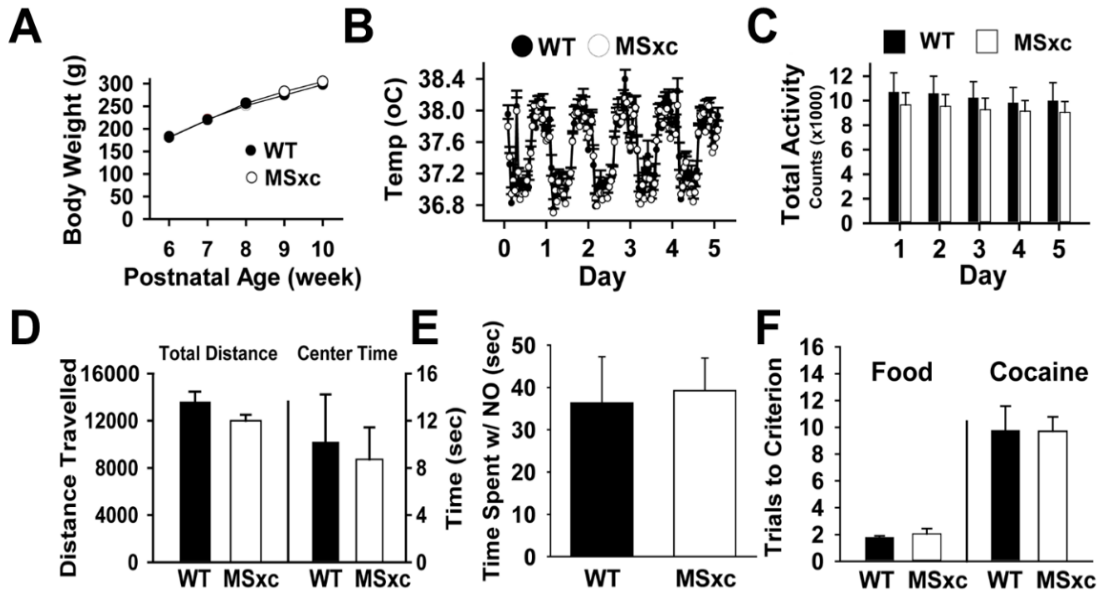


Figure 2-5. Loss of Sxc does not produce widespread impairments in physiology or behavior. **A**, MSxc rats did not display developmental deficits in the form of post-weaning size or growth rates to adulthood. **B,** Central regulation of metabolism as reflected by absolute or diurnal changes to core body temperature was unaltered in MSxc rats. **C-D**, MSxc rats display normal levels of activity in familiar and novel environments. **C**, Telemetry measures of general activity in the home-cage over a five-day period was not altered by genotype. **D**, Activity levels and time spent in the center zone of a novel environment were also similar between WT and MSxc rats. **E,** Simple recognition memory as assessed by comparing time spent with a novel object did not differ between WT and MSxc rats. **F,** Evidence that the capacity to form simple associations was unaltered in MSxc rats also involved a lack of a genotypic effect on the number of trials needed to acquire a food or cocaine-reinforced operant task. Special thanks to Dr. SuJean Choi's Lab for B-C.

$p>0.05$; day $F_{7,91}=2.759$, $p<0.05$). Additionally, there were no genotypic effects on total distance or time spent in the center of an open field arena (Figure 2-5D; n=12/genotype; total distance $t_{22}=1.60$, $p>0.05$; center time $t_{22}=0.306$, $p>0.05$) or novel object recognition (Figure 2-5E; n=9/genotype; $t_{16}=0.216$, $p>.05$). Furthermore, MSxc rats did not differ from WT in trials needed to reach completion criterion when tested in associative operant tasks reinforced with sucrose (Figure 2-5F; n=19-31/genotype; $t_{48}=0.84$, $p>.05$) or cocaine (n=13/genotype; $t_{24}=0.903$, $p>.05$). These data demonstrate that MSxc rats do

not appear to display maladaptive traits in physiology, basic memory tasks, and simple associative learning.

To investigate whether maladaptive disruptions in cognition were present in the MSxc rat, we first employed a Pavlovian-conditioned approach (PCA) paradigm (Figure 2-6). PCA, also referred to as autoshaping, has been used to model the process by which discrete stimuli are learned to be predictive of a desired outcome, acquire motivational properties, and subsequently influence behavior (Robinson, Yager, Cogan, & Saunders, 2014). The rationale for this approach is that PCA allows us to assess the degree of “top-down” versus “bottom-up” regulation of behavior i.e. behavior guided by a calculation of discriminating relevant stimuli in accordance with previously acquired knowledge about the probability of an outcome versus sensory input readily impacting behavioral output, respectively (E. K. Miller & Cohen, 2001; Sarter, Givens, & Bruno, 2001). In PCA procedures, CS+ (reward-predictive conditioned stimulus) approach is viewed as a “bottom-up” driven behavior due to the incentive and motivational value placed on CS+ by the subject, even though this behavior is not required for reward delivery (Sarter & Phillips, 2018).

To ascertain the ability for the subjects to learn that the presentation of CS+ is predictive of a reward delivery, tray entries, CS approaches and the latency to enter were analyzed. A two-way repeated measure ANOVA was used to determine genotypic differences, differences across the training session, and any interaction between these variables on a within-subject basis. Regarding CS+ tray entries (Figure 2-7A; n=9,10/genotype) we report a main effect of

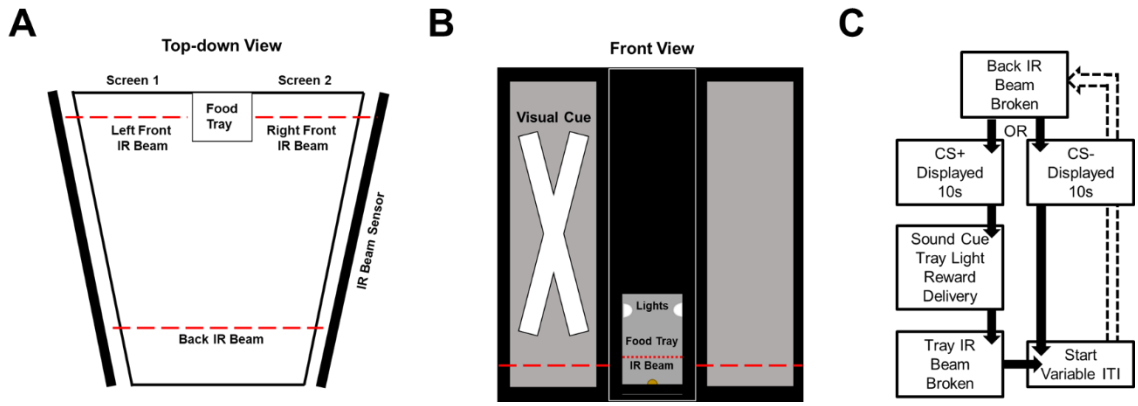


Figure 2-6. Pavlovian conditioned approach experimental design. A, Top- down view of the Campden touch screen behavioral chamber. **B,** Front-view of the behavioral chamber with two rectangular screens spanning the floor to ceiling flanking the reward tray. The conditioned stimulus (CS) is a large visual X on the screen **C,** Flowchart for the training paradigm. Approach toward CS+ is not required for reward delivery.

session ($F_{4,68}$=34.227, $p<0.05$) with no effect of genotype ($F_{1,68}$=1.108, $p>0.05$) nor an interaction between these variables ($F_{4,68}$=1.492, $p>0.05$). Notably, there was an increase in tray entries by sessions 2-5 relative to session 1 (Tukey, $p<0.05$). This increase did not occur to the same extent when CS- was presented, as the effect of session was restricted to an escalation between session 1 and 4 (Figure 2-7B; genotype, $F_{1,68}$=0.847, $p>0.05$; session, $F_{4,68}$=3.465, $p<0.05$; genotype x session; $F_{4,68}$=0.563, $p>0.05$; Tukey, $p<0.05$). Additionally, there was an effect of session on tray entry latency with no effect of genotype nor an interaction. (Figure 2-7E; genotype, $F_{1,68}$=3.965, $p>0.05$; session, $F_{4,68}$=17.378, $p<0.05$; genotype x session; $F_{4,36}$=0.535, $p>0.05$). By sessions 3, 4, and 5, subjects were significantly faster at entering the tray (Tukey, $p<0.05$). Collectively, these data demonstrate that both genotypes were able to associate reward delivery in the tray with CS+ and not CS-.

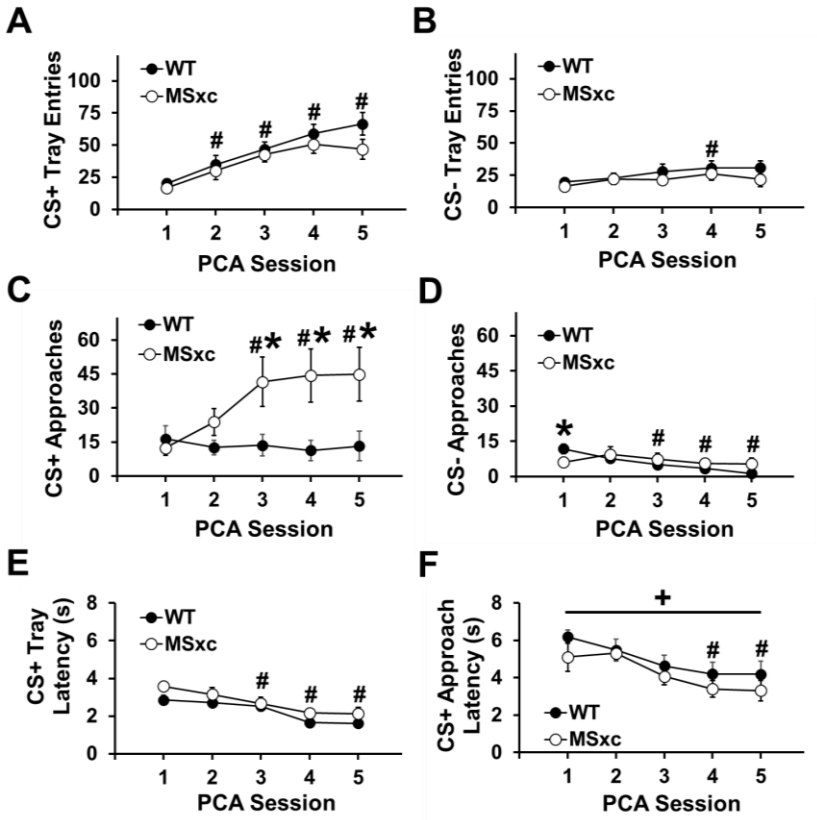


Figure 2-7: Loss of Sxc does not alter associative learning but increases CS+ approach behavior during PCA training. A-B, Data depicts total reward tray entries during the presentation of CS+ or CS- respectively. **C-D,** Total approaches toward the screen during the presentation of CS+ or CS- respectively. **E,** Amount of time prior to the first reward tray entry when CS+ is presented. **F,** Latency to approach CS+ during presentation. Two-way repeated measure ANOVA; # effect of session relative to session 1, + effect of genotype independent of session, * effect of genotype within the given session $p<0.05$.

Next, genotypic differences in CS+ approach behavior, a measure of bottom-up regulation of behavior, was analyzed. Regarding CS+ approaches during presentation (Figure 2-7C), we report main effects of session ($F_{4,68}=4.539$, $p<0.05$) and genotype ($F_{1,68}=5.162$, $p<0.05$) with a significant interaction between these variables ($F_{4,68}=6.571$, $p<0.05$). Notably, there was an increase in CS+ approaches among MSxc rats in sessions 3, 4, and 5 relative to session 1 in addition to an effect of genotype relative to WT within sessions 3-5 (Tukey,

p<0.05). Regarding CS- approaches, there were main effects of session (Figure 2-7D; $F_{4,68}$=4.345, p>0.05) but not genotype ($F_{1,68}$=0.247, p>0.05) with an interaction between these variables ($F_{4,68}$=2.656, p>0.05). Intriguingly, WT rats increased their approaches toward CS- by sessions 3-5 relative to session 1 and WT rats approached CS- more than MSxc rats during session 1 (Tukey, p<0.05) Furthermore, there was an effect of session on CS+ approach latency with no effect of genotype nor an interaction (Figure 2-7F; Two-way ANOVA; genotype, $F_{1,79}$=1.113, p>0.05; session, $F_{4,79}$=2.837, p<0.05; genotype x session; $F_{4,79}$=0.653, p>0.05). Rats approached CS+ more rapidly by sessions 4 and 5 relative to session 1 (Tukey, p<0.05). Notably, rats that did not approach CS+ had no latency measurement so they were excluded from this specific analysis. These data demonstrate that MSxc rats displayed increased CS+ approach behavior as PCA training progressed yet both genotypes approached CS+ faster as training progressed.

Having established genotypic differences in CS+ approach during the PCA training sessions, rats were then tested in an omission phase whereby CS+ approach behavior prevented the delivery of a reward. Omission is meant to test whether the CS+ approach behavior can be inhibited due to negative punishment, a form of top-down cognitive control (E. K. Miller, 2000). We hypothesized that punishment would likely impact CS+ approach but not tray entries. Indeed, tray entries when CS+ was presented (Figure 2-8A), revealed a main effect of session ($F_{4,68}$=4.780, p<0.05) with no effect of genotype ($F_{1,68}$=3.429, p>0.05) nor an interaction between these variables ($F_{4,68}$=0.0296,

p>0.05). Notably, there was an escalation in tray entries by sessions 4 and 5 relative to session 1 (Tukey, p<0.05). A similar phenomenon occurred when CS- was presented (Figure 2-8B; genotype, $F_{1,68}$=1.609, p>0.05; session, $F_{4,68}$=3.582, p<0.05; genotype x session; $F_{4,68}$=0.155, p>0.05) with a small escalation in tray entries by sessions 4 and 5 relative to session 1. Regarding CS+ approaches during presentation (Figure 2-8C), we report a main effect of session ($F_{4,68}$=7.633, p<0.05) an effect of genotype ($F_{1,68}$=6.187, p<0.05) with no significant interaction between these variables ($F_{4,68}$=1.707, p>0.05). Notably, there was a reduction in CS+ approaches independent of genotype in sessions 3, 4 and 5 relative to session 1 (Tukey; p<0.05). This did not occur with CS- approaches (Figure 2-8D; genotype, $F_{1,68}$=0.523, p>0.05; session, $F_{4,68}$=0.870, p>0.05; genotype x session; $F_{4,68}$=0.723, p>0.05). The net effect of this prolonged CS+ approach behavior in MSxc rats was a greater number of missed rewards across all sessions (Figure 2-8E; genotype, $F_{1,68}$=5.859, p<0.05; session, $F_{4,68}$=8.317, p<0.05; genotype x session; $F_{4,68}$=0.832, p>0.05). The number of missed rewards decreased by sessions 3,4, and 5 relative to session 1 (Tukey, p<0.05). Furthermore, the cumulative number of missed rewards during omission testing was greater in MSxc rats (Figure 2-8E; t-test, t_{17}=-2.421, p<0.05). These data demonstrate that both genotypes continue to escalate their tray entries and reduce approaches during CS+ presentation over the course of omission testing, but MSxc rats have a prolonged increase in CS+ approach behavior relative to WT which results in more missed rewards.

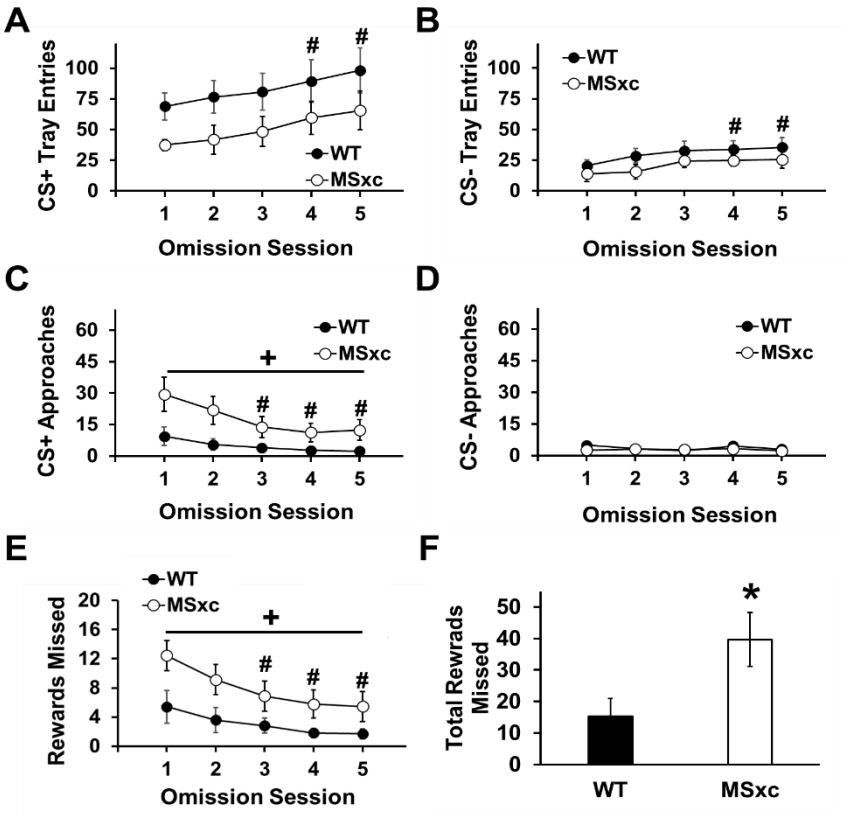


Figure 2-8: CS+ approach behavior is maintained even when punished in MSxc rats during omission testing. A-B, Data depicts total reward tray entries during the presentation of CS+ or CS- respectively. **C-D,** Data depicts total cue approaches during the presentation of CS+ or CS- respectively. **E,** Data depicts the number of rewards missed during the omission testing sessions with a cumulative count presented in **F**. Two-way repeated measure ANOVA; # effect of session relative to session 1, $p<0.05$; + effect of genotype independent of session, $p<0.05$. *students t-test; $p<0.05$. Two-way repeated measure ANOVA; # effect of session relative to session 1, $p<0.05$; + effect of genotype independent of session, *students t-test; $p<0.05$. $p<0.05$.

Alternatively, PCA data can be expressed using the PCA score which incorporates all dependent variables described so far (see methods for description of the score calculation). The PCA score is on a scale of +1 to -1 which correspond to behavior being predominantly directed toward CS+ or the reward tray respectively. These phenotypes are referred to as sign- and goal-tracking respectively (Meyer et al., 2012). During PCA training, there was an

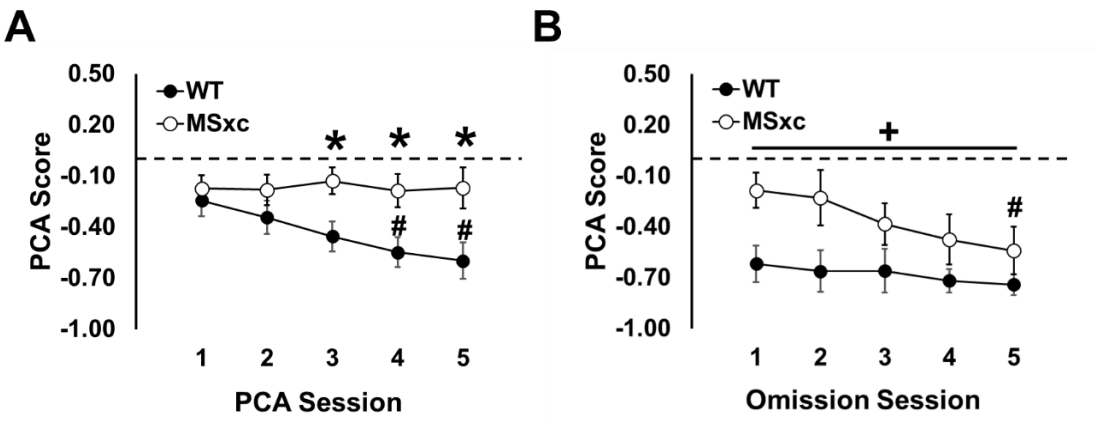


Figure 2-9: Loss of Sxc produces an upward shift in PCA score. A-B, Data depicts PCA scores during PCA training and omission testing, respectively. Please see methods for PCA score calculation. Two-way repeated measure ANOVA; * effect of genotype within the given session, $p<0.05$; + effect of genotype independent of session, $p<0.05$; # effect of session relative to session 1 $p<0.05$.

effect of session (Figure 2-9A; $F_{4,68}=3.076$, $p<0.05$) genotype ($F_{1,68}=5.925$, $p<0.05$) and a significant interaction between these variables ($F_{4,36}=3.147$, $p<0.05$). By session 3,4, and 5, MSxc rats had a significantly higher PCA score relative to WT (Tukey, $p<0.05$). Additionally, the PCA score of WT rats decreased by sessions 4 and 5 relative to session 1 whereas the PCA score of MSxc rats was not impacted by session (Tukey, $p<0.05$). During omission, there were effects of session and genotype with no interaction between these variables (Figure 2-9B; genotype, $F_{1,68}=4.899$, $p<0.05$; session, $F_{4,68}=4.026$, $p<0.05$; genotype x session; $F_{4,68}=1.183$, $p>0.05$). Indeed, PCA score increased by session 5 relative to session 1 independent of genotype (Tukey, $p<0.05$). These data demonstrate that the PCA score for MSxc rats is shifted upward relative to WT which corresponds to greater CS+ directed behaviors. Collectively, the PCA and omission data demonstrate genotypic differences in stimulus-associated

behavior which persists even when rewards are lost which is indicative of a disruption in executive control over behavior.

One interpretation of the prolonged CS+ approach behavior in MSxc rats during omission testing is that MSxc rats have impaired behavioral flexibility. To test, we utilized a maze-based attentional set-shifting paradigm to test MSxc rats' ability to flexibly adapt behavioral strategy to obtain a sucrose reward (Figure 2-10A, n=6-7/genotype). The impact of genotype depended on the trial (Figure 2-10B; Two-way ANOVA, genotype x phase: $F_{2,22}=3.78$, $p<0.05$). Consistent with previous data, there was no effect of genotype during the simple discrimination phase ($t_{11}=0.348$, $p>0.05$). However, MSxc rats needed significantly more trials to reach criterion during the extra-dimensional shift phase ($t_{11}=0.3.83$, $p<0.05$). and intra-dimensional shift phase ($t_{11}=2.96$, $p<0.05$). This phenomenon results from a significant increase in incidence of perseverative (Figure 2-10C; $t_{11}=3.39$, $p<0.01$) but not regressive errors (Figure 2-10D; $t_{11}=0.86$, $p>0.05$) made by MSxc rats during the second and third phase of testing. These data demonstrate that MSxc rats display behavioral inflexibility independent of disruptions in simple associative learning due to perseveration of a behavioral strategy that is no longer reinforced.

To further examine the degree of disrupted executive function in MSxc rats, we employed a Kamin fear blocking paradigm which assesses rats' capacity to regulate attention towards stimuli while learning about threats (Figure 2-11A,

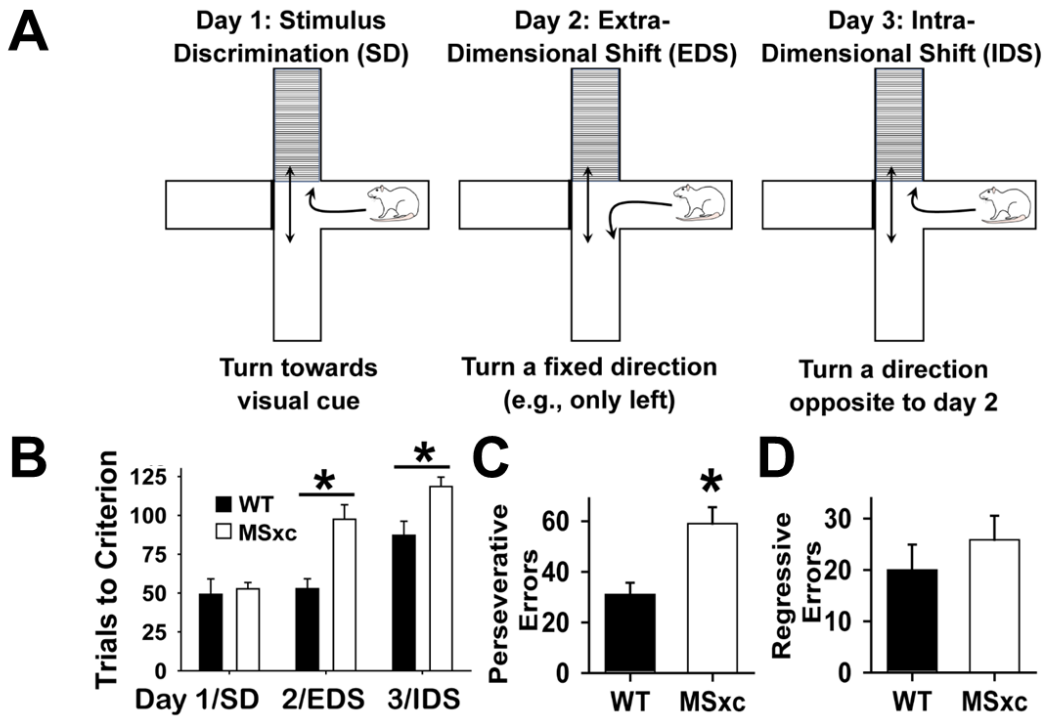


Figure 2-10. Loss of Sxc activity impairs cognitive flexibility A, A schematic illustrating the experimental stages of the attentional set-shifting paradigm in which the number of trials needed to solve a novel maze task is assessed. **B,** The number of trials required to meet criteria for each of the three test sessions is depicted. The impact of genotype depended on the trial. There was no effect of genotype on day 1, which required subjects to use stimulus discrimination (SD) to identify the arm of the maze baited with food. MSxc rats needed significantly more trials to reach criterion on day 2, which required an extradimensional shift (EDS) to solve the task. MSxc rats also needed more trials to reach criterion on day 3, which required an intra-dimensional shift (IDS) to solve the task. **C,** On days 2 and 3, MSxc rats displayed more perseverative errors. **D,** The number of regressive errors on days 2 and 3 did not differ between WT and MSxc rats. *p<0.05 t-test.

n=8/genotype). This procedure creates conditions whereby an association between a novel cue (Cue-B) and shock is prevented or “blocked” by co-presentation of a previously established predictor of shock (Cue-A). Hence, the presence of Cue-A eliminates the predictive value of the novel cue (Cue-B). When exposed to Cue-B on the test day, WT rats did not display an increase from baseline in conditioned freezing but did freeze in response to a novel Cue-D (Figure 2-11B; $F_{2,14}$=10.73, p<0.001; Tukey HSD versus baseline, p<0.05). In

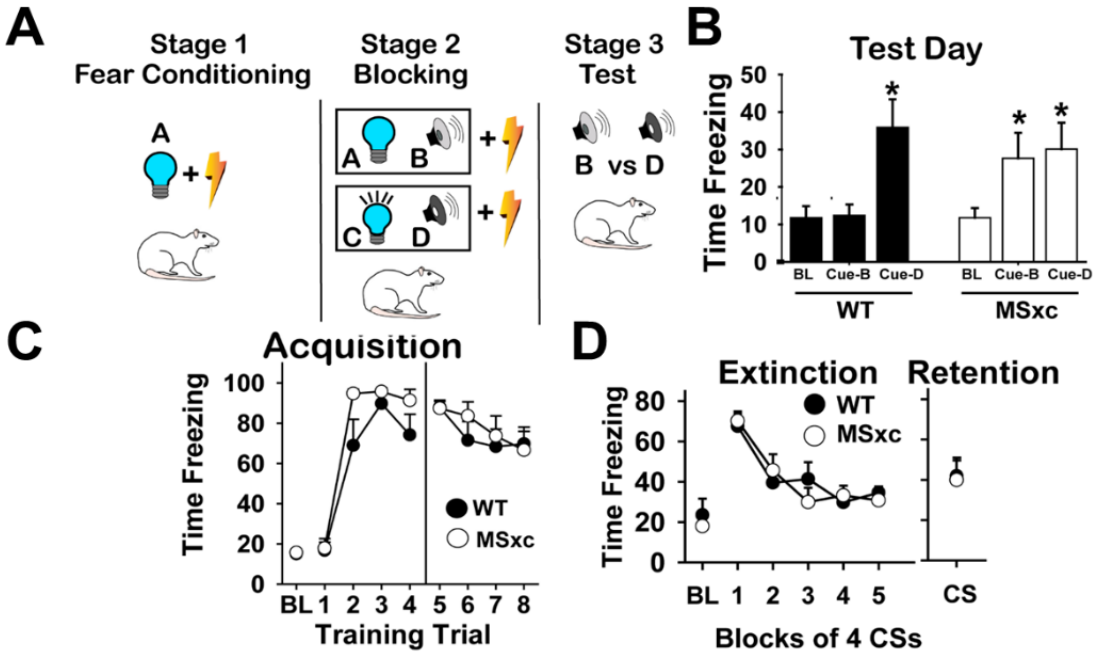


Figure 2-11. Loss of Sxc activity impairs the gating of associative processes. **A,** Schematic illustrating the experimental stages of the Kamin blocking procedure. The procedures are designed so that the co-presentation of an established shock-predictive cue (Cue-A) will block a novel cue (Cue-B; "blocked stimulus") from being paired with shock during Stage 2 and, therefore, from eliciting conditioned freezing on the test day. **B,** Freezing behavior during the test day as measured during baseline (BL) or following exposure to Cue-B or Cue-D. WT rats display conditioned freezing to Cue-D, but not to the "blocked" stimulus, Cue-B. MSxc rats displayed similar levels of freezing to Cue-D and Cue-B, indicating impaired Kamin blocking. **C-D,** Freezing behavior during the acquisition and extinction of fear conditioning. Two-way ANOVA; *effect of cue relative to baseline Tukey $p<0.05$. Special thanks to Dr. Marieke Gilmartin's Lab for this set of experiments.

contrast, MSxc rats displayed a lack of Kamin blocking since these rats displayed a similar amount of conditioned freezing to Cue-B and Cue-D (Figure 2-11B; $F_{2,14}=6.94$, $p<0.01$; Tukey versus baseline, $p<0.05$). This effect was not due to genotypic differences in the acquisition (Figure 2-11C; genotype x trial: $F_{8,12}=1.18$, $p>0.05$; genotype main effect $F_{1,14}=1.70$, $p>0.05$) or extinction of fear

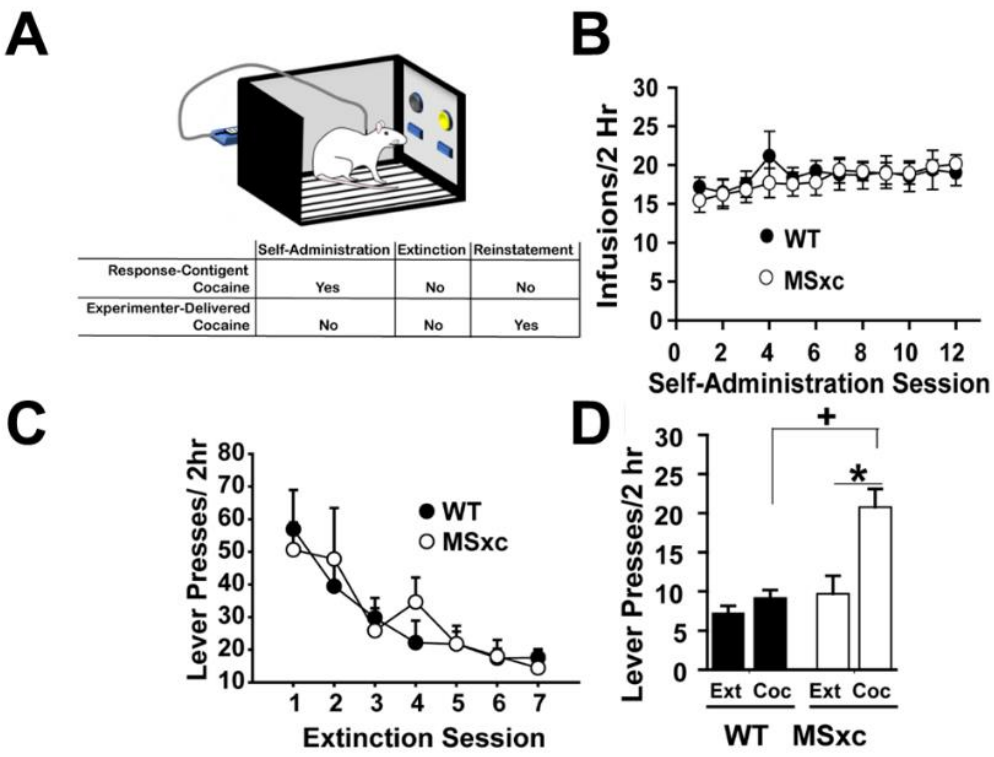


Figure 2-12: Loss of Sxc increases relapse vulnerability. A, Diagram depicting the operant chamber set up and experimental design of the cocaine self-administration model. **B,** Data depicts number of infusions of cocaine received during the maintenance phase (0.5mg/kg/infusion). **C,** Extinction training phase whereby lever presses no longer yielded a cocaine infusion **D,** Reinstatement test phase whereby a subthreshold dose of cocaine was delivered by the experimenter (3 mg/kg I.P). Two-way ANOVA; * Effect of treatment within genotype; $^{+}$Effect of genotype within treatment, Tukey $p<0.05$.

conditioning (Figure 2-11D; $F_{5,70}=0.77$, $p>0.05$; genotype main effect $F_{1,14}=0.52$, $p>0.05$) or in the retention of extinction memories (Figure 2-11D; $F_{1,14}=0.37$, $p>0.05$). These data lend further evidence towards MSxc rats having executive dysfunction.

The maladaptive associative learning and inflexible, perseverative patterns of behavior seen in MSxc rats are observed in a variety of human disorders including substance abuse (Ersche, Roiser, Robbins, & Sahakian, 2008; Keiflin & Janak, 2015; Steinberg et al., 2013). To determine whether a loss of Sxc activity

impacts reinforced and non-reinforced drug-seeking, we employed a cocaine self-administration paradigm which includes three main phases: self-administration, extinction of lever responding, and reinstatement of seeking (Figure 2-12A). Importantly, rates of cocaine self-administration did not differ across genotype (Figure 2-12B; genotype x session: $F_{11,264}$=0.816, p>0.05; genotype: $F_{1,24}$=0.052, p>0.05). Additionally, responding under extinction conditions, whereby the lever was no longer reinforced with cocaine, did not differ across genotype (Figure 2-12C; genotype x session: $F_{1,24}$=0.611, p>0.05; genotype: $F_{1,24}$=0.005, p>0.05). However, during the cocaine-primed reinstatement test, MSxc rats displayed higher levels of responding relative to their last extinction trial that otherwise did not reinstate responding in WT rats (Figure 2-12D; t_{11}=3.26, p<0.05 WT versus MSxc during cocaine test). These data demonstrate that loss of Sxc function does not alter self-administration of cocaine or extinction learning. However, MSxc rats display increased vulnerability for relapse to an otherwise sub-threshold dose of cocaine which suggests a lack of executive control over a behavior that no longer elicits a rewarding outcome.

Having established that loss of Sxc impairs executive function, we next sought to determine how loss of Sxc impacts the tripartite synapse which may in part help illuminate the observed cognitive dysfunction. In order to ascertain molecular disruptions in the glutamate system of MSxc rats, we chose a key component from each compartment of the tripartite synapse that Sxc has been shown to interact with; the pre-synaptic domain, the astrocyte, and the post-

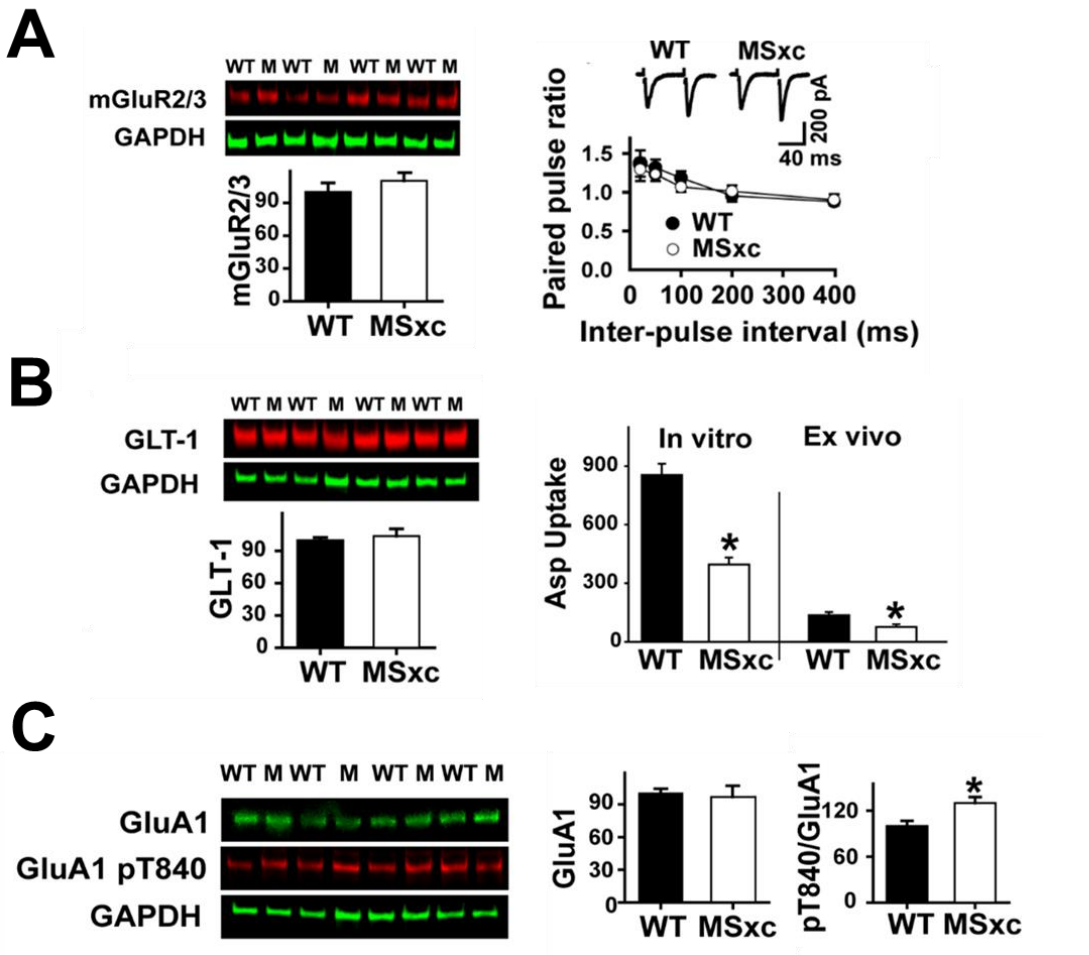


Figure 2-13. Loss of Sxc alters components of the tripartite synapse. A, NAc protein levels of the presynaptic protein mGluR2/3 and paired-pulse ratio. **B,** NAc protein levels of the astrocytic glutamate transporter GLT-1 and ^{3}H-D-aspartate uptake in both NAcc tissue (ex vivo) and cultured astrocytes (in vitro), as a measure of excitatory amino acid transporter function. **C,** Western blots of the postsynaptic protein GluA1 and GluA1 phosphorylated at threonine 840 ($GluA1_{pT840}$). * $p<0.05$, t-test.

synaptic domain. In the pre-synaptic domain, we found no difference in group 2 metabotropic glutamate receptor expression (mGluR2/3; Figure 2-13A; n=8/genotype; $t_{14}=0.967$, $p>0.05$) which corresponded to no change in pre-synaptic release probability as measured by paired-pulse ratio (Figure 2-13A). Additionally, GLT-1 expression was unchanged in MSxc rats (Figure 2-13B; n=8/genotype; $t_{14}=0.26$, $p>0.05$). However, when EAAT function was measured

by ^{3}H-D-Aspartate uptake, MSxc NAcc tissue had significantly lower uptake relative to WT (Figure 2-13B; n=15-18/genotype; $t_{31}=2.69$, $p<0.05$). This disruption was also present in cultured astrocytes (Figure 2-13B; n=11-12/genotype; $t_{21}=6.61$, $p<0.05$). Furthermore, post-synaptic changes were also investigated; total GluA1 expression was unchanged in MSxc rats (Figure 2-13C; n=7-8/genotype; $t_{14}=0.967$, $p>0.05$) but there was a significant increase in GluA1 phosphorylation at threonine residue 840 (pT840; Figure 2-13C; $t_{13}=2.93$, $p<0.05$). These data demonstrate the presence of impaired EAAT function and GluA1 hyper-phosphorylation in the NAcc of MSxc rats independent of changes in pre-synaptic release of glutamate.

Given the molecular and functional changes in GluA1 and EAATs, we hypothesized that excitatory output from the NAcc would be increased in MSxc rats. Miniature excitatory post-synaptic currents (mEPSCs) were measured from medium spiny neurons (MSNs n=15-17/genotype) projecting to the substantia nigra (SN; Figure 2-14A,C) and ventral pallidum (VP; Figure 2-14B,D). The frequency ($t_{26}=2.7$, $p<0.05$) and amplitude ($t_{26}=2.35$, $p<0.05$) of mEPSCs were increased in MSxc SN MSNs which correlated with a leftward shift in intervals and rightward shift in amplitude cumulative probability plots. Additionally, mEPSCs were unaltered in VP MSNs (Frequency, $t_{30}=0.01$, $p>0.05$; amplitude, $t_{31}=0.52$, $p>0.05$). These data suggest that loss of Sxc produces an enhancement of synaptic strength in an efferent specific manner and consequently highlight a role for Sxc in regulating AMPAR function and gating excitatory output for specific neural networks.

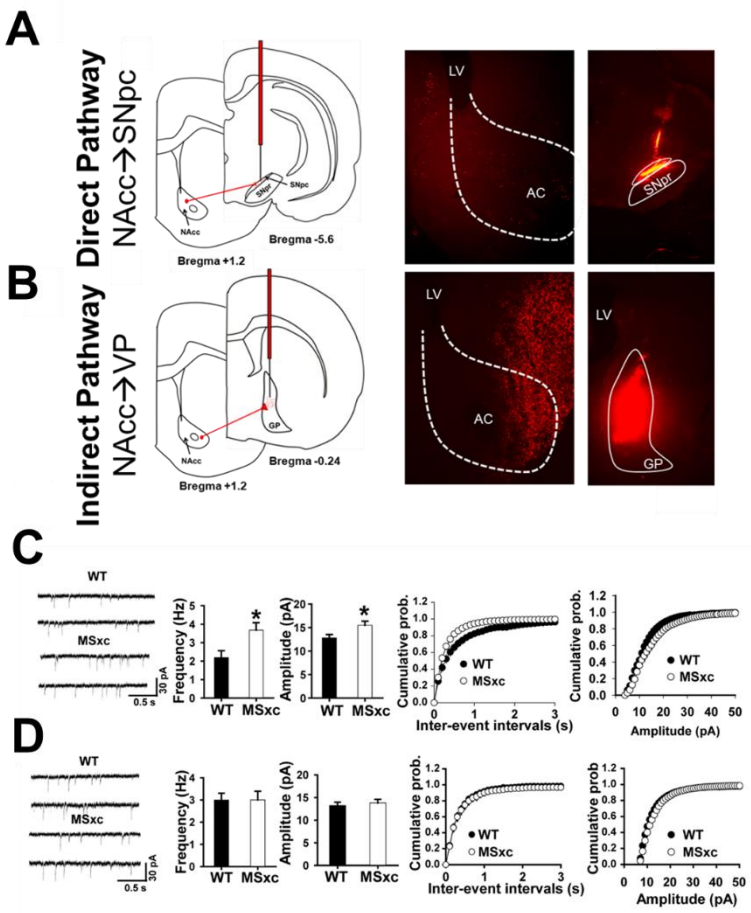


Figure 2-14. Loss of Sxc enhances basal excitatory neurotransmission. A, Diagram depicting rhodamine latex microsphere injection targeting the substantia nigra pars compacta (SNpc); center- expression of rhodamine fluorescent cells in the NAcc; right- validation of the injection site. **C,** Left- diagram depicting rhodamine latex microsphere injection targeting the globus pallidus (GP); center- rhodamine fluorescent cells in the NAcc; right- validation of the injection site, hitting the majority of the GP. **B,** Sample traces from WT and MSxc SNpc projecting and **D,** GP projecting MSNs. (Right) Cumulative probability plots for inter-event intervals and amplitude of mEPSCs recorded from the respective efferent. * T-test $p<0.05$. Special thanks to Dr. Qing-song Liu for C-D.

DISCUSSION

The purpose of these studies was to test the possibility that the acquisition of Sxc was a key event in achieving the signaling complexity needed for executive function and cognition in vertebrate species. We hypothesized that disrupting Sxc function would be sufficient to disrupt glutamate neurotransmission and produce a rat with maladaptive behavioral traits. *We found that eliminating Sxc increased the likelihood that environmental stimuli, and previously learned associations about them, would be more likely to elicit an*

inappropriate behavioral response. Despite altering behavioral phenotypes in PCA, set shifting, fear blocking, and cocaine reinstatement tasks, the loss of Sxc did not disrupt exploratory behavior, basic learning and memory, or general physiology. This suggests that the role of Sxc in cognition is discrete and restricted to executive functions such as reversal learning, attentional gating, and behavioral control. This degree of cognitive dysfunction in a natural environment would likely put MSxc rats at a significant disadvantage, making them maladapted to their environment.

Furthermore, loss of Sxc produced discrete disruptions in excitatory signaling in the NAcc. We found that loss of Sxc broadly impaired EAAT function in astrocytes. Additionally, AMPAR were hyperphosphorylated at residue T840 which corresponded to an enhancement in mEPSCs in the direct but not indirect pathway from the NAcc. Notably, these changes were independent of any change in pre-synaptic glutamate release. The significance of this pathway specific enhancement is evidenced by the direct pathway being important for driving motivated behavioral responses (Bromberg-Martin, Matsumoto, & Hikosaka, 2010; Lobo et al., 2010). This may in part explain why cognitive dysfunction was observed in MSxc rats, due to an inability to effectively gate excitatory output in this pathway. However, more work will be needed to determine if dampening this pathway would be sufficient to rescue the cognitive deficits.

These findings reveal that the existence of evolutionarily new glutamate-release mechanisms could enable the isolation and therapeutic targeting of

signaling needed for specialized domains of brain function, such as cognition. Indeed, there is growing evidence that compounds that increase Sxc activity such as n-acetylcysteine have significant therapeutic potential in pre-clinical and clinical studies of cognitive disorders such as drug addiction, binge eating disorder, trichotillomania, and obsessive compulsive disorder (Amen et al., 2011; J. E. Grant, Odlaug, & Kim, 2009; Hurley et al., 2016; Oliver et al., 2015; Tomko et al., 2018). Importantly, these studies highlight the efficacy of n-acetylcysteine and its tolerability in patients due to a lack of significant side effects which supports the discrete role of Sxc in brain function and behavior, thus making it an excellent therapeutic target.

The potential for Sxc to confer important benefits to brain complexity and behavioral capabilities should be evident in the evolutionary history of *Slc7a11*/xCT. The Sxc-subunit xCT first appeared some point after the divergence of protostomes and deuterostomes (Lewerenz et al., 2013), which indicates that Sxc is a relatively newer adaptation in animals. Our key findings from our phylogenetic analysis were that the majority of the xCT protein coding sequence was under significant negative selective pressure in vertebrates, and the sequence has gained novel kinase regulatory sites. Specifically, our SLAC analysis, which detects amino acids that were under selective pressure, found that 70% of the amino acids had a greater degree of synonymous than non-synonymous mutations occurring within their codons. This means that while mutations did occur, they were more likely to encode the same amino acid. This demonstrates that selective pressures resisted changes in the sequence, such

that if a non-synonymous mutation were to occur, it would be rapidly removed from the gene pool through purifying selection. In other words, the mutation would make the organism maladapted to their environment. Importantly, the SLAC analysis did not detect evidence of positive selective pressure, e.g. a gain of an adaptive mutation, in the xCT sequence.

While there were no sites under positive selective pressure, further inspection of the sequence revealed the gain of regulatory sites. The three sites were S26, S185, and S481 and have varying degrees of conservation in vertebrates. For example, S26 is more represented in primates than in birds, reptiles, fish, or other mammals whereas S481 is unique to mammals. This suggests these sites were involved in speciation, perhaps through providing some functional advantage. However, our SLAC analysis did not conclude that these regulatory sites were positively selected for. While advantageous mutations are of interest, detecting them is difficult considering that synonymous mutations and negative selection are the predominant evolutionary forces (M. Kimura, 1968; Motoo Kimura, 1983; Kreitman, 2000). Given this, there are two principle interpretations that can be drawn about the significance of the regulatory sites. First, our statistical methods used to determine selective pressure are limited by the available sequence data. While we analyzed 115 vertebrate species, there are many more species whose xCT sequence is not known. By adding more data, it is possible that sites under positive selection would arise. On the other hand, acquisition of these sites may have been due to random genetic drift that just happened to become stabilized within certain

species. With either interpretation, further work is needed to elucidate the biological relevance of these novel regulatory sites in xCT.

Collectively, the phylogenetic signature of xCT suggests it is a fundamental protein to vertebrates; organisms without it have maladaptive cognitive traits. While our phylogenetic analysis failed to find evidence for mutations that provided an adaptive advantage, this approach still revealed novel regulatory sites that were gained during vertebrate evolution. If we can characterize the functional relevance of these regulatory sites, we will be able to understand their contribution to cognitive ability. Using this information, we could investigate the possibility that polymorphisms that alter these sites make humans more susceptible to cognitive disorders. In support, the phosphorylation of S26 has been shown to regulate Sxc function and dysregulation of this site may contribute to the psychotomimetic effects of phencyclidine in rats, a pre-clinical model of schizophrenia (McClatchy et al., 2016). Thus, these findings are consistent with the possibility that disrupted function of evolutionarily new genes expressed by astrocytes may underlie cognitive impairments.

Intriguingly, while xCT itself is a relatively recent adaptation on the evolutionary timeline (Lewerenz et al., 2013), its ancestral homologs in invertebrates share similar functional characteristics and suggests that it has been critical for cognitive processing for millions of years. In support, it is accepted that invertebrate species such as *c. elegans* and *drosophila* can learn conditioned approach responses and are able to successfully adapt behavior in response to changes in the environment to improve survivability (Foley,

Marjoram, & Nuzhdin, 2017; Zhao, Khare, Feldman, & Dent, 2003). Importantly, they express a putative xCT homolog named genderblind (gb), which has approximately 36-45% sequence homology to mammalian xCT and contains the essential cystine residue (C158) allowing it to dimerize to 4F2hc (Augustin, Grosjean, Chen, Sheng, & Featherstone, 2007). The function of gb is strikingly like mammalian xCT. Its function is to maintain extracellular glutamate which desensitizes neuronal glutamate receptors and dampens excitatory output. Its role in cognitive processing is to regulate how olfactory chemical stimuli influence approach behavior. Consequently, *drosophila* lacking gb more readily engage in homosexual mating behaviors due to misinterpretation of sexual olfactory stimuli, which is inherently maladaptive (Grosjean, Grillet, Augustin, Ferveur, & Featherstone, 2008). Furthermore, deletion of gb is not inherently fatal, which suggests that gb/xCT have maintained a discrete role in cognitive processing for millions of years. Thus, while xCT is considered a relatively recent adaptation, it has maintained some of the functional and qualitative characteristics of its ancestral homologs.

While a narrower manipulation of Sxc such as a conditional knockout in adulthood may have been an alternative approach to investigate certain aspects of our hypothesis, we felt the global knockout was the only way in which broad questions about the role of Sxc in mammalian cognition could be addressed. While an argument could be made for the observed cognitive dysfunctions being the result of developmental perturbations, previous work from our lab demonstrates that acute pharmacological disruptions in adulthood produced

similar deficits in behavioral flexibility to those seen in the MSxc rat (Lutgen et al., 2014). Additionally, our global knockout approach is similar to what was used to explore the functional role of the *Dlg* orthologs in cognitive processing in mice (S. G. Grant, 2016). Furthermore, If the global knockout approach was not used, we would not have learned that loss of Sxc is not inherently fatal and is void of any obvious developmental impairments which would be suggestive of it being involved in fundamental brain functions that support life, and consequently make it a risker therapeutic target. Lastly, because Sxc is present in the amygdala, hippocampus, prefrontal cortex and NAcc (Baker, McFarland, Lake, Shen, Tang, et al., 2003; Lutgen et al., 2014), which are all brain regions involved in cognitive processing, an acute knockdown approach in each region coupled to all of our behavioral measures would have been laborious and it does not control for the possibility that Sxc dependent signaling in these other brain regions could compensate for disruption in another. Thus, our global knockout approach was appropriate for the questions we sought to answer, which revealed that Sxc has a discrete role in cognition and is not critical for fundamental brain functions that sustain life.

PCA has been used to model the process by which stimuli are learned to be predictive of a desired outcome, acquire motivational properties, and subsequently influence behavior (Robinson et al., 2014). In canonical models of PCA, an operant chamber is outfitted with retractable levers which serve as the CS and are predictive of a food reward delivery. Over repeated pairings, the lever extension triggers a conditioned approach and appetitive response to the lever

as it has become imprinted with the incentive and perhaps emotional value of the food reward (Meyer, Cogan, & Robinson, 2014). A common observation is that two distinct behavioral phenotypes emerge during training; sign tracking and goal tracking, which correspond to the rats' propensity to guide appetitive behavior towards the CS or towards the reward tray, respectively. These phenotypes are characterized by the PCA score, which is on a scale of +1 (sign tracking) to -1 (goal tracking) with an intermediate phenotype at 0 (Meyer et al., 2012). Over repeated training, sign tracking behavior becomes the predominant phenotype, with little to no tray entries during the presentation of the CS. A key distinction relative to our experiments is the use of a retractable lever rather than a visual stimulus as the CS. Notably, our WT rats over the course of training display almost a complete lack of CS approach behavior but do increase tray entries across training whereas MSxc rats increase CS approach *in addition to tray entries* that persists even when punished for CS approach. The lack of approach behavior in WT rats was surprising but it is consistent with reports that demonstrate conditioned approach is reduced when the CS cannot be physically interacted with (Cleland & Davey, 1983; Meyer et al., 2014). Because of this, our ability to observe the degree to which the CS influences behavior in MSxc rats relative to WT was enhanced. Furthermore, this suggests that the PCA score and the categorization of sign- versus goal-trackers can be unreliable due to it being heavily influenced by experimental parameters.

CS+ approach behavior exhibited by MSxc rats may be indicative of inappropriate associative learning and an impairment in executive function.

Importantly, reward delivery is not contingent on the rat approaching CS+. It is possible that during the initial phases of training, MSxc rats formed an inappropriate association between their CS+ approach behavior and reward delivery. Given that CS+ approach continued to increase across sessions, it is likely that MSxc rats were learning that their approach is what was causing reward delivery. In this way, CS+ approach is viewed as a “bottom-up” driven behavior due to the incentive and motivational value placed on CS+ by the subject (Sarter et al., 2001; Sarter & Phillips, 2018). When CS+ approach was punished during omission, MSxc rats continued to engage the CS+ which is indicative of a lack of behavioral control or impaired “top-down” regulation of behavior. The prefrontal cortex in particular is necessary when behavior needs to be guided or altered by changes in internal or environmental circumstances, such as when the probability of an outcome is changing (E. K. Miller & Cohen, 2001). These findings have translational validity, as rats that display heightened CS+ directed behavior during PCA prefer cocaine over natural rewards when given a choice (Tunstall & Kearns, 2015) and are more vulnerable to relapse when exposed to drug-associated stimuli (Versaggi, King, & Meyer, 2016). Thus, it was not surprising to see that MSxc rats displayed heightened cocaine-primed reinstatement.

Impaired executive function was also observed during the attentional set shifting and fear blocking paradigms. During set shifting, the rat must form the association that the arm of the maze with a visual stimulus is predictive of reward. On subsequent days the rat has to adapt a new strategy to obtain reward

(always turn a given direction) as the visual cue is no longer absolutely predictive of reward. Because the environmental circumstances and probability of a rewarding outcome is changing, the executive centers such as the prefrontal cortex are necessary to appropriately guide behavior (E. K. Miller & Cohen, 2001). Likewise, for Kamin fear blocking, the executive centers are required efficiently direct focal attention toward environmental stimuli that are predictive of danger while blocking out ones that are not (Diamond, 2013; Posner & DiGirolamo, 1998). Expectedly, WT rats did not show fear to a novel stimulus that was co-presented with a stimulus that was previously learned to be predictive of danger. On the other hand, MSxc rats did show fear which suggests they lack the ability to effectively gate extraneous sensory information. Given our behavioral findings, MSxc rats appear to have impaired executive function. As a result, *environmental stimuli, and previously learned associations about them, are more likely to elicit an inappropriate behavioral response in MSxc rats.*

Importantly, MSxc rats are able to extinguish drug seeking and fear response behavior, which may challenge the assertion that Sxc is involved in gating how conditioned responses elicit future behavioral responses. Extinction, by definition, is a learning phenomenon whereby responding to CSs decreases when the outcome is omitted (Quirk & Mueller, 2008). Extinction learning has three phases: acquisition, consolidation, and retrieval. The acquisition phase is the initial learning phase whereby responses begin to decline due to a lack of reinforcement. This learning is then consolidated into a long-term memory which can be recalled during retrieval (e.g. reinstatement) to elicit a behavioral

response. As demonstrated by both the fear conditioning and cocaine self-administration paradigms, there is no genotypic difference in the acquisition or consolidation of extinction memory. The lack of genotypic difference in acquisition of extinction may be explained by this phase being a *new* learning phenomenon similar to the initial conditioning phase. Specifically, we know that MSxc rats do no struggle with forming simple associations between CSs and outcomes and therefore we should expect them not to struggle with extinction acquisition. Additionally, CS responding during extinction training steadily declines over time in both genotypes which suggests that the new extinction memory is being remembered or consolidated.

Intriguingly, the retrieval of extinction memories is impacted by loss of Sxc, but only under certain circumstances. In particular, retrieval of extinguished fear memory is not impacted by loss of Sxc whereas retrieval of cocaine seeking is, as evidenced by the increase in seeking in MSxc rats following a cocaine challenge. This discrepancy in retrieval may exist due to the nature of the retrieval trigger, i.e. cocaine versus a light stimulus. Specifically, it is plausible that the cocaine rather than light stimulus has a greater impact on the already enhanced direct pathway neurons present in the MSxc rats and thus is more likely to facilitate the reinstatement of seeking. Further work is needed to explore the discrepancy in the genotypic differences in extinction memory retrieval but it highlights how discrete the loss of Sxc is, even within the same dimension of learning. Furthermore, these extinction training phenomena suggest that *Sxc is involved in influencing how learned associations elicit changes in behavioral*

response during tasks that require active attentional or working memory allocation.

The major technical advancements of this work are the novel transgenic GFAP:Lck-eGFP rat to aid in studying the astrocyte transcriptome and the MSxc rat to study the importance of Sxc function in neurophysiology and behavior. Importantly, these animals allowed us and will hopefully allow others to advance our knowledge of the interplay between astrocytes, glutamate signaling, neural network activity and cognition. In support, FACS data collected from the GFAP:Lck-eGFP rat is consistent with experiments conducted *in situ* to investigate the expression of xCT in astrocytes (Ottestad-Hansen et al., 2018). An important note is that while some xCT expression is present in the GFP- sample, it is possible that we did not collect every astrocyte present in the sample. Notably, our sorting parameters were designed to exclude neurons from eGFP+ samples rather than isolate every astrocyte. As a result, the eGFP- sample also contained a very small number of astrocytes that were not completely disassociated from other cells.

Additionally, the GFAP:Lck-eGFP rat revealed that GFAP expression can vary across brain regions. We observed the highest GFP expression in subcortical areas such as the thalamus as well as in the midbrain, brainstem, and cerebellum. The GFP signal in the neocortex, hippocampus, and striatum was comparatively lower. This is consistent with work done in human brain samples that demonstrate regional differences in GFAP expression, with subcortical regions expressing more GFAP than the neocortex (Torres-Platas, Nagy, Wakid,

Turecki, & Mechawar, 2016). While the reason underlying these differences remains unknown, the regional differences in GFAP expression appear to be phylogenetically conserved among rats and humans.

The molecular and cellular changes resulting from loss of Sxc, while relatively minor, appear to be consistent with previous findings. It was surprising to not see a change in mGluR2/3 expression or its ability to regulate pre-synaptic release probability given previous work supporting group 2 mGluRs in relaying Sxc dependent signals (Baker et al., 2002). However, the xCT null mutant mice did not display changes in pre-synaptic plasticity, consistent with our findings (Y. Li et al., 2012). Additionally, pT840/GluA1 density was increased in hippocampal synapses of xCT knockout mice which corresponded to an increase in spontaneous and evoked EPSCs (Williams & Featherstone, 2014). We found a similar increase in the NAcc of our MSxc rats and established that this phenomenon may occur in a pathway specific manner given that enhanced mEPSCs were found only in the direct pathway.

The mechanism underlying how only the direct pathway is influenced by loss of Sxc or how Sxc can regulate GluA1 phosphorylation remains unresolved. We speculate that there could be differences in the proximity of Sxc to NAcc efferents or that the neuronal receptor responsible for receiving Sxc derived glutamate that is regulating GluA1 is differentially expressed or no longer being activated. In support of the latter interpretation, NMDAR activation can lead to

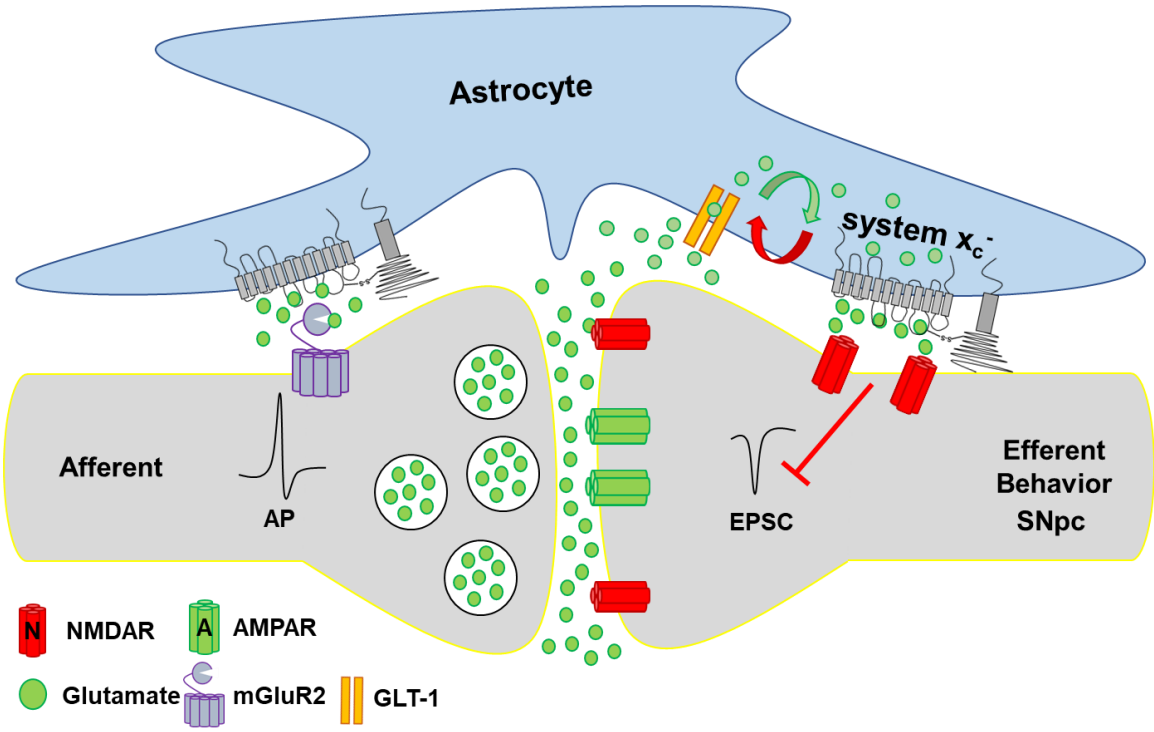


Figure 2-15: Model of the tripartite synapse in the NAcc. Our findings demonstrate a link between astrocyte to neuron signaling via Sxc being a critical component of neural network regulation and ultimately executive control over behavior. Previous work suggests Sxc communicates through pre-synaptic mGluRs to gate synaptic release while this work demonstrates Sxc can gate AMPA receptor signaling, likely through activation of extrasynaptic NMDARs. This will be explored later.

dephosphorylation of T840/GluA1 (Delgado et al., 2007). Given the lateral diffusion capabilities of NMDARs (Groc et al., 2004), they may be in a position to receive non-synaptic glutamate signals from astrocytes. Indeed, NMDAR activation via glutamate release from astrocytes produces slow inward post synaptic currents (SICs). What makes SICs unique are their slow kinetics and dependency on the proximity of an astrocyte (Fellin et al., 2004; Kovacs & Pal, 2017). Our data suggest that Sxc may signal through NMDARs to influence GluA1, and this will be directly tested in subsequent experiments.

Sxc and EAATs such as GLT-1 have been suggested to be strongly co-regulated (Knackstedt, Melendez, & Kalivas, 2010). While it was surprising that we saw no change in GLT-1 expression, we chose to investigate changes in D-aspartate uptake, which is a more general measure of EAAT function. Not surprisingly we saw a reduction in D-aspartate uptake in MSxc tissue and cells, which is consistent with the co-regulation hypothesis of Sxc and EAATs. A limitation of this study is that only a single concentration of D-aspartate was used. Future experiments will be aimed at investigating changes in transport kinetics which may help elucidate the nature of this apparent reduction in function.

Understanding brain function is one of the greatest challenges in modern science; it also has among the highest potential to reduce the burden of human disease. However, a paradigm shift in neuroscience may be needed for dramatic advances in understanding the human brain. Given our findings, perhaps the description of astrocytes as mere metabolic support cells is emerging as an example of an overly simplistic and possibly inaccurate perspective that is limiting the progress of modern neuroscience research. Incorporating advances and approaches from artificial intelligence, evolution, and other disciplines have the potential to transform efforts to understand the healthy and diseased brain to uncover novel therapeutic strategies. Our data are consistent with the possible organization of glutamate signaling whereas evolutionarily newer glutamate-release mechanisms expressed by astrocytes, e.g. Sxc, have a restricted, specialized set of functions in regulating neural circuits and cognition. Hence,

further work into evolutionarily newer forms of intercellular signaling may enable the selective manipulation of discrete domains of brain activity, such as cognition and better equip us to treat the brain’s most debilitating diseases.

CHAPTER III:
ENDOGENOUS FACTORS THAT REGULATE SXC

INTRODUCTION

The previous chapter establishes Sxc as a critical component in cognitive processing. Sxc appears to be necessary for gating the influence that environmental stimuli and their learned associations have on eliciting an appropriate behavioral response. Considering this, the next question to address is *how* Sxc is involved in this process. *Specifically, there is a need to determine what molecular factors are influencing Sxc function and the mechanisms underlying Sxc regulation of neural circuits in a manner relevant for cognitive control.*

When we consider the NAcc tripartite model, incoming stimulus information is largely conveyed by the afferent neurons which transmit their information to the direct pathway to drive a behavioral output. An unresolved question is how and when astrocytes are recruited for the regulation of neural circuits. Theoretically, if the complexity of the incoming information is high, neural networks may recruit astrocytes to aid in processing of information to increase efficiency and expedite an appropriate response. If this is true, then perhaps neurons release a factor signaling to astrocytes that they are needed to assist with cognitive processing.

Class	Total Analyzed	CaMKII/MSK1 R-L-P-S[26]	CaMKII/MSK1 R-I-M-S[481]	GSK-3 S-M-S-V-S[185]
Cartilaginous Fishes	1	0 (0%)	0 (0%)	1 (100%)
Fringe-finned Fishes	1	0 (0%)	0 (0%)	1 (100%)
Ray-finned Fishes	14	0 (0%)	0 (0%)	5 (36%)
Reptiles	6	3 (50%)	0 (0%)	6 (100%)
Birds	39	31 (79%)	0 (0%)	39 (100%)
Mammals	42	19 (45%)	33 (79%)	42 (100%)
Primates	12	12 (100%)	12 (100%)	12 (100%)
TOTAL	***115***	***65 (57%)***	***45 (39%)***	***106 (92%)***

Figure 3-1: Conservation of xCT consensus phosphorylation sequences among vertebrates. Sequences were deemed conserved so long as the base consensus sequence was retained; R-X-X-S and S-X-X-X-S for CaMKII/MSK1 and GSK-3β respectively.

The previous chapter established that xCT acquired novel kinase regulatory sites during vertebrate evolution which may have allowed neural factors to signal through astrocytes and regulate Sxc function which in turn, allowed Sxc to impact encoding of environmental information. There are three proposed regulatory sites on the human sequence of xCT that are conserved in rats: S26, S185, S481. These serine residues match consensus sequences for CaMKII/MSK1 and GSK-3 (Figure 3-1). Intriguingly, the degree of conservation among these sequences is different, with the GSK-3β site having a 92% conservation rate and the two MSK1 sites having rates of 57% and 39% (S26 and S481 respectively). Furthermore, the emergence of S26 occurs in some reptiles and most birds, but expression falls in mammals but becomes stoic in primates. Likewise, S481 emerges first in mammals and is present in all primates, suggesting that these regulatory sequences were recently acquired and may be downstream targets of a variety of intracellular signaling cascades triggered by multiple neural factors. This leads to the questions; which neural factors regulate Sxc and what are the functional changes resulting from these biophysical adaptations?

In order to investigate the contribution of neural factors to the function of Sxc, we chose to explore three possible sources of influence: the post-synaptic neuron, pre-synaptic neuron, and hormones and select a molecular factor that might target one of these sites. First, the post-synaptic neuron could be contributing to the regulation of Sxc. Recent studies support a role of endocannabinoid mobilization in striatal efferents which act on the cannabinoid-1 receptor (CB1R) in adjacent astrocytes which drives glutamate release at homo-typic, hetero-synaptic circuits. This glutamate activates neuronal mGluRs and consequently enhances synaptic glutamate signaling and thus output power (Martin et al., 2015). Importantly, the mechanism that releases glutamate from astrocytes in this study was not investigated, which questions whether Sxc is involved. Furthermore, CB1R activation has been shown to regulate GSK-3β which targets S185 of xCT (Ozaita, Puighermanal, & Maldonado, 2007).

Regarding the pre-synaptic terminal, the neuropeptide $PACAP_{1-38}$ (shorthand PACAP) is co-released with glutamate and can influence the expression and function of glutamate transporters in astrocytes such as Sxc (Kong et al., 2016; Resch et al., 2014) and GLT-1 (Figiel & Engele, 2000) in addition to neuronal NMDARs and AMPARs (Toda & Huganir, 2015; Yaka, He, Phamluong, & Ron, 2003). However, the rate in which PACAP influences Sxc and GLT-1 is slow, as it takes days of application in vitro to see changes in expression and function. Rapid effects of PACAP on Sxc function remain to be studied and these findings would have important implications for the potential to rapidly influence neural networks. Furthermore, PACAP has been shown to

influence MSK1, which targets S26 and S481 of xCT (Butcher, Lee, Cheng, & Obrietan, 2005).

Lastly, hormones could potentially be influencing Sxc function. A recent study has suggested that Sxc is a resilience factor for stress by observing that a reduction in the expression of xCT was present in the ventral hippocampus of mice exposed to chronic restraint stress for 21 days. Remarkably, when n-acetylcysteine was orally administered during the last 3 days of stress exposure, xCT expression was restored and social interaction behavior improved (Nasca et al., 2017). This finding calls into question whether stress related hormones such as corticosterone are influencing the expression or function of Sxc. Furthermore, glucocorticoids have been shown to activate MSK1 and drive its translocation from the nucleus to the cytosol which would put it in position to regulate S26 or S481 of xCT.

Conducting a series of *in vitro* experiments using cultured astrocytes, we investigated the ability for endocannabinoid, PACAP, and corticosterone signaling to influence the function of Sxc through cystine uptake or cystine-evoked glutamate release. These findings will help illuminate how neural and hormonal factors regulate astrocytes through modulating Sxc function and in turn regulate neural circuits to encode incoming stimulus information.

MATERIALS AND METHODS

Cell Culture: Astrocyte cultures were generated from postnatal day-3 rat pups. The striatum was dissected and dissociated using 0.25% trypsin EDTA (Gibco) and cultured in 75 cm^2 flasks in a humidified incubator at 37°C under 95% O_2 5%

CO_2 in Eagles minimum essential medium (Gibco) supplemented with 5% fetal bovine serum/5% horse serum (Atlanta Biologicals), Glutamax (Gibco), and antibiotics/antimycotics (Gibco). To remove debris and non-astrocytic glia, flasks were agitated, and the resulting mono-cell layer was resuspended with 0.25% trypsin EDTA. Cells were counted by hand via a cytometer and seeded in 24-well plates coated with poly-D-lysine and laminin at a density of 200,000 cells per well.

Glutamate release assay: Striatal astrocytes (DIV14) were incubated for 30 minutes at 37^{o}C in Na^{+}-free buffer containing the following: 116 mM choline chloride, 13.4 mM MgSO4, 1.68 mM KH_2PO_4, 2.34 mM $CaCl_2$, 5.49 mM dextrose, 11.9 mM HEPES, 0.2% choline bicarbonate, titrated to pH: 7.4 with CeOH. This buffer was used to prevent Na^{+}-dependent uptake of glutamate. L-cystine (12.5 or 200 μM) were applied to drive cystine-glutamate exchange by Sxc. These concentrations were chosen because they approximate the required cystine for Km and Vmax of xCT transport. Corticosterone-HBC and PACAP treatments were applied for 30 minutes at which point media samples (100 μl) were collected for subsequent glutamate analysis using HPLC. Cells were then dissolved in 0.5% SDS and total protein for each well was quantified using the BCA method.

Glutamate HPLC: The concentration of glutamate was quantified by comparing peak areas from samples and external standards using HPLC coupled to fluorescence detection. A 10 μl sample underwent pre-column derivatization with orthophthaladldehdye (OPA) in the presence of 2-mercaptoethanol using a

Shimadzu LC10AD VP autosampler. Chromatographic separation was achieved using a Kinetex XB C-18 (50 x 4.6 mm, 2.6 μm; Phenomenex) and a mobile phase consisting of 100 mM Na_2HPO_4, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% acetonitrile at a pH of 6.04. Glutamate was detected using a Shimadzu 10RF-AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively. Glutamate content for each sample was normalized to total protein in the respective well and depicted as a net change from baseline. The data was fit using the Michaelis-Menten equation to determine Km and Vmax.

^{14}C-L-Cystine Uptake Assay: Cultures were rinsed with 1x HBSS media prior to application of vehicle [1x HBSS, 1% FBS, 0.1% dimethylsulfoxide (DMSO), 200nM ^{14}C-L-Cystine] or WIN55 [1x HBSS, 1% FBS,1μM WIN55,212, 200nM ^{14}C-Cystine] treatment solutions. Plates were incubated at 37^{o}C for 20 minutes followed by three rinses with 1x HBSS. Cells were lysed with 300μL of 0.5% sodium dodecyl sulfate (SDS) per well. A 200μL aliquot was taken for scintillation count and a 25μL aliquot was taken for BCA assay.

Data analysis: All experiments were analyzed using a two-way ANOVA with a Tukey post-hoc test. Data are presented as mean ± SEM.

RESULTS

An *in vitro* radiolabeled cystine (^{14}C-L-cystine) uptake assay was utilized to ascertain the sufficiency for a CB1R agonist (WIN55,212) to rapidly enhance Sxc-dependent cystine uptake (Figure 3-2; n=11-12/genotype). A two-way ANOVA revealed a main effect of treatment ($F_{1,42}$=7.225, $p<0.05$), genotype

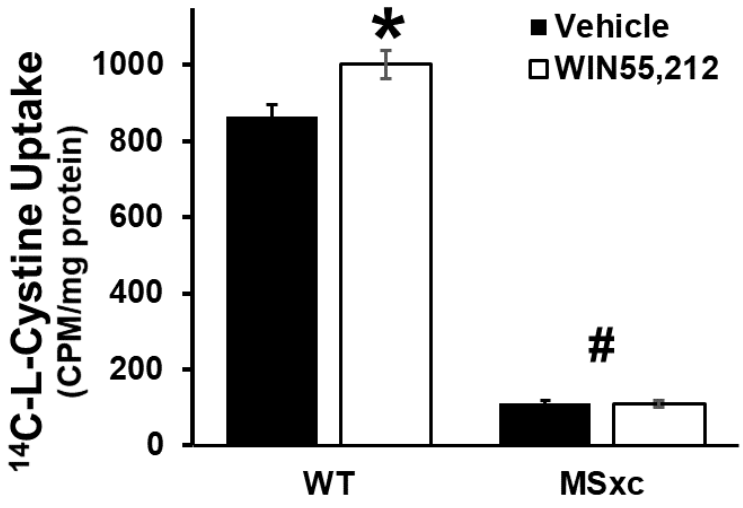


Figure 3-2: CB1R agonism enhances Sxc dependent cystine uptake. Data depicts mean ± SEM radioactivity of WT and MSxc cell samples treated for 20 minutes with radiolabeled L-cystine with or without the CB1R agonist WIN55,212. L-cystine was applied at a concentration of 200 nM. Two-way ANOVA, Tukey post-hoc test; # effect of genotype within treatment; *effect of treatment within genotype $p<0.05$.

($F_{1,42}$= 1073.739, $p<0.05$), with a significant interaction between these variables ($F_{1,42}$= 7.475, $p<0.05$). Post-hoc tests revealed a significant increase in ^{14}C-L-cystine uptake in WIN55,212 treated WT but not MSxc cells (Tukey, $p<0.05$). These data demonstrate that CB1R agonism rapidly enhances Sxc dependent cystine uptake within 20 minutes and loss of Sxc greatly reduces cystine uptake.

Next, an *in vitro* cystine-evoked glutamate release assay was conducted to determine the effect of PACAP application on extracellular glutamate in WT and MSxc cells (Figure 3-3; n=6-12/genotype). One cystine concentration were used in this experiment, 12.5 μM, which approximates the Km of Sxc mediated transport. A two-way ANOVA revealed a main effect of genotype ($F_{1,40}$= 275.645, $p<0.05$) and treatment ($F_{3,40}$= 4.492, $p<0.05$) with a significant

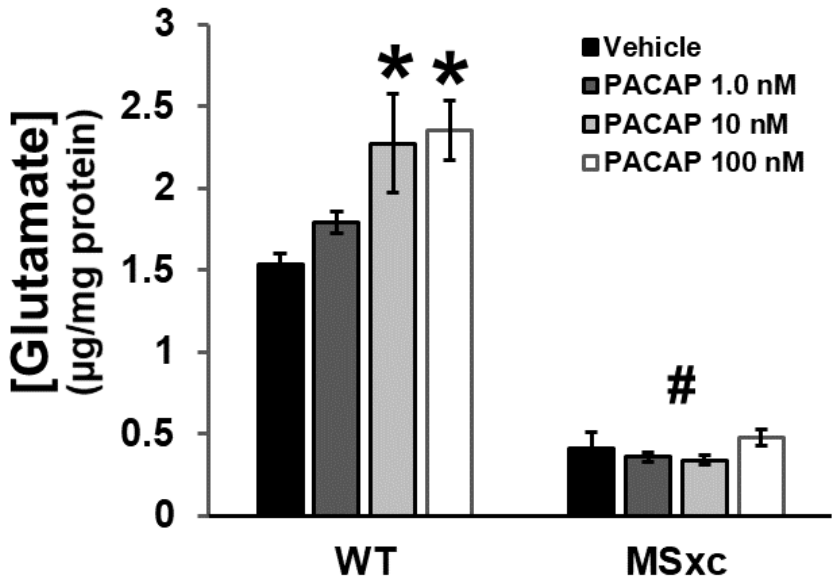


Figure 3-3: PACAP dose dependently increases glutamate release via Sxc. Data depicts mean ± SEM extracellular glutamate evoked by a 30-minute cystine (12.5 µM) and $PACAP_{1-38}$ treatment. Two-way ANOVA, Tukey post-hoc test; # effect of genotype within treatment; *effect of treatment within genotype p<0.05.

interaction between these variables ($F_{3,40}$= 4.003, p<0.05). There was a large reduction in extracellular glutamate in MSxc cells within each PACAP treatment group (Tukey, p<0.05). Within WT cells, PACAP dose-dependently increased extracellular glutamate (0 nM vs 10 and 100 nM; Tukey, p<0.05). This phenomenon did not occur in MSxc cells (Tukey, p<0.05). These data demonstrate that PACAP can rapidly increase extracellular glutamate in an Sxc-dependent manner and that loss of Sxc greatly reduces extracellular glutamate. Lastly, an *in vitro* cystine-evoked glutamate release assay was conducted to determine the effect of corticosterone application on extracellular glutamate in WT cells (Figure 3-4; n=3-4/treatment). Two cystine concentrations were used in this experiment: 12.5 and 200 µM, which estimate the Km and Vmax of Sxc mediated transport, respectively. A two-way ANOVA revealed an interaction between treatment and cystine ($F_{2,16}$= 3.762, p<0.05). There was a dose dependent increase in extracellular glutamate resulting from corticosterone

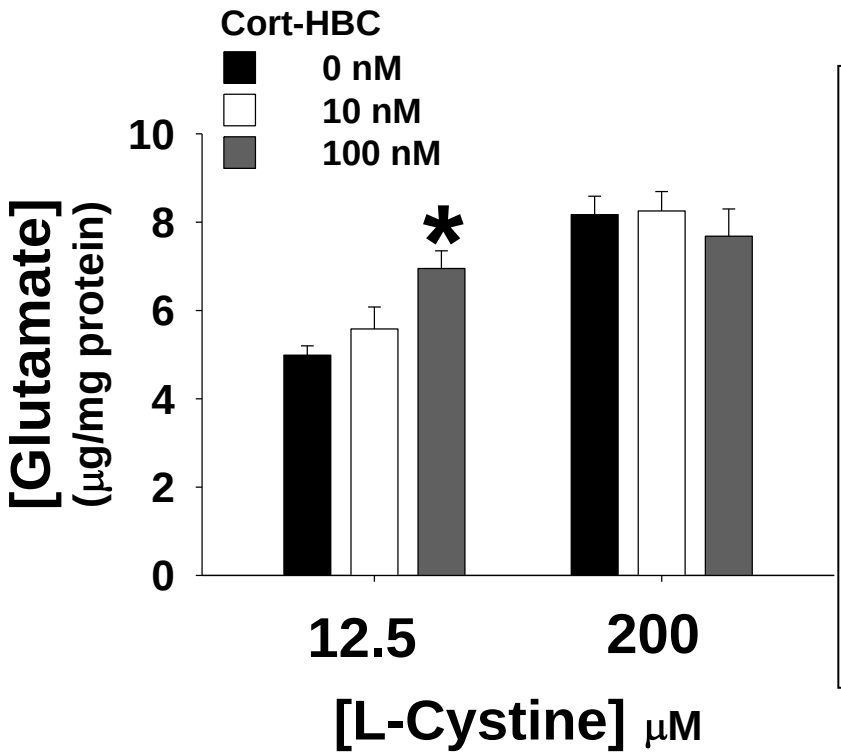


Figure 3-4: Corticosterone enhances glutamate release form astrocytes in a cystine-dependent manner. Data depicts mean ± SEM extracellular glutamate evoked by a 30-minute cystine and corticosterone treatment. Two-way ANOVA, Tukey post-hoc test; *effect of treatment within cystine treatment $p<0.05$.

treatment (0 vs 100 nM) within the 12.5 μM cystine treatment group (Tukey, $p<0.05$). This phenomenon did not occur in the 200 μM cystine treatment group (Tukey, $p>0.05$). These data demonstrate that corticosterone significantly enhances cystine-evoked glutamate release when rates of uptake are near the Km but not Vmax of transport.

DISCUSSION

These data establish three potential sources of influence for Sxc function: the pre-synaptic neuron, post-synaptic neuron, and hormonal milieu. We chose to investigate the endocannabinoid, PACAP, and corticosterone signaling due to the likely possibility that they are influencing one or more of the putative regulatory sites on xCT: S26 and S481 which are regulated by MSK1 and S185 which is regulated by GSK-3β. Furthermore, these experiments provide the first

evidence that PACAP, corticosterone and CB1R agonism can *rapidly* influence Sxc function. Given the predicted kinase regulatory sites on xCT via MSK1 and GSK3, there is a clear need to investigate how these factors might be influencing these kinases. Additionally, the extent to which these factors influence behavior through Sxc remain to be studied, but given the findings in the MSxc rat, it is possible that these factors can modify how astrocytes regulate neural networks and behavior.

The impact of corticosterone on Sxc function appears to be specific to the efficiency of transport or Km given that glutamate release was only impacted at 12.5 μM L-cystine. Effectively, this means that Sxc can expel more glutamate at lower cystine concentrations. Unfortunately, it is not currently understood how Sxc does this, but it may be through a biophysical adaptation caused by phosphorylation of the xCT subunit which increases the transport probability of cystine and glutamate. Furthermore, we did not control for effects independent of Sxc, as a negative control such as sulfasalazine treated or MSxc cells were not treated with corticosterone. Future experiments will be aimed at understanding whether the effects seen here are Sxc dependent and if so, how corticosterone may change the phosphorylation of xCT. Given the link between glucocorticoid signaling and activation of MSK1 (Beck et al., 2013), we hypothesize that S26 or S481 are likely impacted by corticosterone.

The impact of corticosterone on Sxc function may have implications for cognitive control as stress can influence performance in behavioral flexibility tasks (Hurtubise & Howland, 2017). Intriguingly, the intensity of the specific

stress paradigm can differentially impact performance in set shifting or reversal learning. Mild stressors such as a brief 30-minute restraint or being placed on an elevated platform prior to testing would improve reversal learning suggesting that stress in this context is providing an adaptive advantage. Conversely, repeated exposure to stressors for a week or more broadly impaired set shifting and reversal learning which suggests that stress over prolonged periods of time produces a maladaptive behavioral phenotype. Because Sxc has been linked to stress resiliency (Nasca et al., 2017) it is feasible that stress hormones such as corticosterone could be affecting Sxc function and consequently changing the behavioral response to acute and chronic stressful stimuli. Future work should be aimed at investigating this link.

Given our experimental design, we cannot determine the effect of CB1R agonism on Sxc kinetics. At best, due to the use of a low concentration of L-cystine (1 μM), we can speculate that the increase in L-cystine uptake seen with CB1R agonist treatment is indicative of a reduction in Km of Sxc transport. Much like with the effect of corticosterone, how CB1R agonism increases the efficiency of Sxc transport is unknown but likely involves a phosphorylation event. Importantly, this finding illuminates Sxc as one of the mechanisms that is involved in CB1R-dependent glutamate release from astrocytes. The finding that CB1R signaling in astrocytes leads to pathway specific regulation of neural circuits via glutamate release mirrors our findings in the first chapter demonstrating that loss of Sxc induces a pathway specific enhancement of excitatory output from the NAcc (Martin et al., 2015). While this paper did not

investigate the glutamate release mechanism downstream of CB1R, we can speculate that one of the targets may be Sxc. Furthermore, CB1R activation has been shown to regulate GSK-3β which targets S185 of xCT (Ozaita et al., 2007). Given this, future experiments aimed at understanding the link between Sxc and the endocannabinoid system are warranted and may reveal how endocannabinoids influence astrocytes to regulate behavioral control.

Like corticosterone, the endocannabinoid system can produce effects on performance in behavioral flexibility tasks, which are suggestive of its link to Sxc function. For example, when the CB1R antagonist AM251 is administered at low doses prior to a spatial to visual cue or visual cue to spatial shift in response strategy, the number of perseverative errors committed were reduced. Intriguingly, higher doses did not produce this effect. Furthermore, when the CB1R agonist HU-210 is administered, there are dose-dependent effects on perseverative errors with low doses reducing and higher doses increasing these errors. Intriguingly, the low dose of HU-210 increased the number of regressive errors made when shifting towards a spatial cue which suggests latent inability to maintain the new behavioral strategy after sufficient successful trials. This may be due to the difficulty in shifting away from the visual cue which has both visual and tactile qualities and makes it a more salient cue relative to spatial cues (Hill, Froese, Morrish, Sun, & Floresco, 2006). Thus, the potential for stimuli and the learned associations about them to influence behavior is in part determined by endocannabinoid signaling. An important follow up experiment will involve

manipulating the endocannabinoid system in WT and MSxc rats to determine the necessity of Sxc in these behavioral changes.

Lastly, the finding that PACAP dose-dependently and rapidly enhances Sxc-dependent cystine-evoked glutamate release from astrocytes mirrors previous findings that long-term incubation increases Sxc expression and function (Kong et al., 2016; Resch et al., 2014). The rapid nature of this enhancement is critical, since for PACAP to rapidly influence behavior, the signaling mechanism needs to be able to quickly modulate Sxc function. Again, we speculate that this rapid regulation likely comes in the form of a phosphorylation event, possibly at one of the serine residues. Notably, there is a link between PACAP signaling and regulation of MSK1 which targets S26 and S481 of xCT (Butcher et al., 2005). Future experiments will investigate the intercellular signaling pathway that links PACAP receptors and Sxc.

The ability for PACAP to influence cognitive control remains understudied. What is currently known about the influence of PACAP on instrumental behaviors comes from a comprehensive behavioral battery conducted on PACAP KO mice (Hattori et al., 2012). In this study there were remarkable increases in locomotor activity and social interaction but no discernable differences in working memory tasks such as forced alternation and left-right discrimination. However, there was an increase in the number of revisiting trials during a radial arm maze task only when the delay period in between trials was high. Unfortunately, the authors did note that some PACAP KO mice died during this test due to food restriction which may have influenced

the outcome. Because of the limitations of the species used, a more direct investigation of the ability for PACAP signaling to regulate cognitive processing in rat is required.

Collectively, these data establish three molecular factors are influencing Sxc function. *The next question that needs to be resolved is what are the mechanisms underlying how neural factors signal through astrocytes and neurons to coordinate neural circuits in a manner relevant for cognitive control?*

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CHAPTER IV:
THE NEUROPEPTDE PACAP COORDINATES NEURON-ASTROCYTE GLUTAMATE SIGNALING IN THE NUCLEUS ACCUMBENS CORE TO ATTENUATE DRUG SEEKING

INTRODUCTION

The previous chapters have established the complexity of intercellular communication in the brain and how the emergence of astrocytes and their ability to release glutamate may have been a critical component involved in mammalian cognition. However, our findings up until this point have been lacking insight into how glutamate signals from astrocytes are encoded by neurons to modify behavior. While the previous chapter suggests that neural and hormonal factors have the capability of influencing Sxc function *in vitro*, there is a disconnect between the production of this signal and the signal transduction mechanism that alters neural network activity *in vivo.* The importance of neuron-astrocyte-neuron communication may be to encode when astrocytes are required for processing of complex information to elicit an appropriate behavioral response. Effectively, this grants neurons the ability to influence astrocyte function and consequently regulate adjacent synapses to either enhance or inhibit excitatory output and behavioral action. Thus, the goal of the following experiments was to investigate how an endogenous neuronal factor regulates Sxc within astrocytes and consequently alters neuronal activity and contributes to cognitive processing.

$PACAP_{1-38}$, (shorthand PACAP) is a particularly interesting factor because of its ability to broadly regulate the glutamate system. Specifically, PACAP has been shown to regulate the expression and function of Sxc and GLT-1 as well as

AMPARs and NMDARs (Figiel & Engele, 2000; Kong et al., 2016; Resch et al., 2014; Toda & Huganir, 2015; Yaka et al., 2003) . Additionally, PACAP levels decrease in the aging brain which correlates with a reduction in cognitive function (Han et al., 2017). However, there are important questions about a potential link between these two outcomes; is it possible for changes in astrocytic glutamate transport to alter post-translational modification of neuronal glutamate receptors and consequently influence excitatory output and cognitive control?

In support of this link, our data in conjunction with others, have established signaling mechanisms involving Sxc and their impact on neuronal signaling and behavior. Sxc can signal through extrasynaptic glutamate receptors within the pre- and post-synaptic domain. In the pre-synaptic domain, Sxc signals through group 2 mGluRs which are $G\alpha_i$ coupled and when activated, attenuate the opening of voltage-gated Ca^{2+} channels consequently preventing vesicular release of glutamate or dopamine (Baker et al., 2002). Post-synaptically, Sxc influences AMPAR function through diminishing the phosphorylation of T840 of GluA1 which is thought to either reduce ion conductance or reduce synaptic localization of AMPAR (Williams & Featherstone, 2014). Although, the mechanism linking dephosphorylation of AMPAR with Sxc activity has not been demonstrated, T840 is decreased by NMDAR activation (Delgado et al., 2007). Given that NMDARs can receive extrasynaptic glutamate signals, it is possible that Sxc may be communicating through these receptors to regulate AMPARs. Furthermore, PACAP signaling in neurons dephosphorylates T840 and this phenomenon is dependent on NMDAR activation (Toda & Huganir, 2015). Thus,

we hypothesize that PACAP may have independent signaling mechanisms in neurons and astrocytes that are linked by glutamate release via Sxc to regulate GluA1.

Considering the link between PACAP, Sxc, NMDARs, and AMPARs, how can these components interact in a way to alter behavior? Importantly, Sxc expression declines following withdrawal from cocaine (Baker, McFarland, Lake, Shen, Tang, et al., 2003), nicotine (Knackstedt et al., 2009), and opioid (Alshehri, Hakami, Althobaiti, & Sari, 2018) intake which produces a drop in extrasynaptic glutamate. This could cause a disinhibition of GluA1 T840 dephosphorylation which in turn could enhance neural outputs in a pathological manner. Consistent with this, chapter 2 illustrated how loss of Sxc function caused a pathway specific enhancement of the direct pathway from the NAcc which has been linked to drug seeking behavior (Lobo et al., 2010). Importantly, the mechanism underlying this reduction in expression is unknown. One possibility is that a neural factor could be regulating Sxc expression and upon cessation of drug consumption, the factor becomes impaired which produces the effect on Sxc. It is possible that PACAP signaling may become dysregulated because of substance abuse, but no evidence exists at this time. However, given the importance of glutamate signaling in the NAcc for the reinstatement of drug seeking and the sufficiency of Sxc to attenuate it, we determined that investigating PACAP signaling in this brain region would be appropriate.

Thus, we tested the hypothesis that PACAP enhances Sxc function and coordinates the reception of its glutamate signal in neurons to regulate neural networks and consequently drug seeking behavior in the NAcc.

MATERIALS AND METHODS

Chemicals: Unless noted, common chemicals and reagents were obtained from Sigma Aldrich.

Animal care and usage: Male Sprague Dawley rats were used for these experiments. Housing conditions and experimental protocols were approved by Institutional Animal Care and Use Committees at the Medical College of Wisconsin or Marquette University, and were carried out according to the US National Institutes of Health guidelines.

Creation of GFAP:Lck-eGFP transgenic rats: The Sleeping Beauty (SB) transposon transgenesis method(Geurts et al., 2010) was used to produce transgenic rats on the Sprague Dawley (Crl:SD) background. Briefly, a GFAP promoter-driven fusion between the plasma membrane targeting myristolation domain of the mouse LCK protein fused to eGFP (Benediktsson et al., 2005) was synthesized (GeneArt) and cloned into a SB transposon vector. The transposon plasmid was co-injected with a source of SB100X transposase mRNA into the pronucleus of fertilized Crl:SD embryos. A breeding colony was established harboring a single copy of the transgene inserted on rat chromosome 13 enabling robust eGFP expression. To confirm expression *in situ*, a rat was deeply anesthetized and transcardially perfused with 4% paraformaldehyde. The brain

was extracted and sliced at 100 µm and GFP signal was visualized using standard fluorescence microscopy.

Creation of MSxc rats: Zinc-finger nucleases (ZFNs) were designed targeting the second exon sequence (TGCTAGCTTTTGTTCgagtcTGGGTGGAACTGCTG) to produce small deletions of a limited number of base pairs in the *Slc7a11* gene, where capital letters represent binding sites for the individual ZFN monomers, on opposite strands. ZFNs were injected into the pronucleus of Sprague Dawley (Crl:SD) rat embryos by pronuclear microinjection of in vitro-transcribed encoding messenger RNAs and the resulting offspring were screened for mutations using a Cel-1 assay and validated by Sanger sequencing as previously described (Geurts et al., 2010) and resulting in single-step, whole-animal disruption of *Slc7a11* (MSxc rats). Deletion of 39 consecutive base pairs (GAGGTCTTTGGTCCCTTGCTAGCTTTTGTTCGAGTCTGG) of exon 2 was confirmed by Sanger sequencing.

Tissue Dissociation for FACS: NAcc tissue was dissected from male GFAP-lck-eGFP or WT Sprague Dawley rats and placed into ice-cold Hanks´ Balanced Salt Solution without Ca^{2+} or Mg^{2+}. Tissue was mechanically and enzymatically dissociated to obtain single-cell suspension using Neural Tissue Dissociation Kit - P (Miltenyi Biotec). 1% BSA, 1mM EDTA and 12.5 U/ml DNase I were added to reduce cell clustering. Cells were incubated with 3 µM Calcein Violet 450 AM Viability Dye (eBioscience) on ice for 10 minutes to stain live cells. An aliquot

from each cell suspension was used to determine relative gene expression in sorted and unsorted (total) cells.

Flow Cytometry: FACS analyses were performed using FACSDiva software 6.1.3 (BD Biosciences). Control samples (tissue obtained from rats lacking the GFAP:Lck-eGFP transgene) were analyzed first to identify the range of endogenous or background fluorescence intensity. In addition, the gating parameters of the flow cytometer were set to isolate live, eGFP+ cells from cellular debris, and cell doublets. Note, the eGFP- samples contain all tissue excluded from the eGFP+ sample and would be expected to include eGFP- cells, cell doublets, fragmented cells, and potentially eGFP+ cells that were not excluded due to the use of strict gating parameters or incomplete cellular disassociation.

RNA amplification and Reverse Transcription: FACS samples were centrifuged (170xg, 5 min) and the supernatant was carefully extracted. Cells were lysed using the cell lysis solution provided within the cDNA Synthesis from Cell Lysates kit (Lucigen). This kit was used as per the manufacturer's instructions to amplify the RNA and reverse transcribe to cDNA for use in PCR or qPCR. Briefly, a T7 RNA polymerase promoter was annealed to the RNA and was reverse transcribed using MMLV reverse transcriptase. *In vitro* transcription was performed, and the RNA product was treated with DNAse and purified using the RNA Clean and Concentrator kit (Zymo). The RNA was then incubated at 70^{o}C and rapidly cooled to 4^{o}C. A final reverse transcription step yielded cDNA for use in downstream applications.

RT-PCR: PCR was conducted using GoTaq DNA polymerase (Promega). Primer sequences were as follows: Slc7a11 (xCT) forward- 5’ AGG GCA TAC TCC AGA ACA CG 3’; Slc7a11 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’; GFAP forward - 5’ GCA GGT GAG GAA GAA ATG GA 3’; GFAP reverse - 5’ TAC GAT GTC CTG GGA AAA GG 3’; RbFox3 forward - 5’ ATC ATA CCA TCG GCC CCA CA 3’; RbFox3 reverse - 5’ GTG AAG CGG CTG TAC CCT CC 3’; ADCYAP1R1 forward – 5’ ATT CTT TCT CCC TCC CCT GA 3’; ADCYAP1R1 reverse – 5’ TGG AGT ACC AAG GGC TGA AC 3’; VIPR1 forward – 5’ AGG GAA AGG ACA GAG CAA CC 3’; VIPR1 reverse - 5’ GGC CTC TAC CAG GAC TTT CC 3’; VIPR2 forward – 5’ GTG CTA CAA GCC CTG GTG TC 3’; VIPR2 reverse – 5’ GGT CAC GCC TAG TGG TTG AT 3’; PACAP forward - 5′ AAC CCG CTG CAA GAC TTC TA 3′; PACAP reverse- 5′ CTT TGC GGT AGG CTT CGT TA 3′.

Western Blotting: NAcc tissue samples obtained from adult WT and MSxc rats (i.e., > 90 days old) underwent mechanical homogenization in a sucrose-based buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.4) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration for each whole cell lysate sample was determined via the bicinchoninic acid assay (BCA). Protein (10 μg) was resolved in a 7.5% polyacrylamide gel and wet transferred to Immobilon PVDF membranes (EMD Millipore). Membranes were immersed for one hour in blocking buffer (LiCOR) prior to overnight application at +4°C with a primary antibody: rabbit anti-pT840 GluA1 (Abcam, 1:3,333), mouse anti-GluA1

(EMD Millipore, 1:1000), rabbit anti-S897 GluN1 (Abcam, 1:1000), and mouse anti GluN1 (Abcam, 1:1000). Secondary antibodies (LiCOR) goat anti-rabbit IR 680 DYE (1:15000) donkey anti-mouse IR 800 DYE (1:20,000) were applied for 1 hour at room temperature prior to imaging (Odyssey Fc Imaging System, LiCOR). Band intensity was quantified using LiCOR Image Studio software and the resulting data are presented as the target protein band intensity divided by the reference protein band intensity.

Cell Culture: Astrocyte cultures were generated from postnatal day 3 rat pups. Briefly, the striatum was dissected and dissociated using 0.25% trypsin EDTA (Gibco) and cultured in poly-D-lysine and laminin treated 75cm^2 flasks in a humidified incubator at 37°C under 95% O_2 5% CO_2 in Eagles minimum essential medium (Gibco) supplemented with 5% fetal bovine serum/5% horse serum (Atlanta Biologicals), Glutamax (Gibco), and antibiotics/antimycotics (Gibco). To remove contaminating glia and dead cells, the flasks were agitated, and the resulting mono-cell layer was resuspended with 0.25% trypsin EDTA. Cells were counted by hand via a cytometer and seeded in 24-well plates coated with poly-D-lysine and laminin at a density of 200,000 cells per well.

D-Aspartate Uptake Assay: Striatal astrocytes (DIV14) were incubated for 30 minutes at 37ºC in HEPES buffered Hanks Balanced Salt Solution. A range of D-aspartate concentrations (0, 12.5, 50, 200, 800 μM) were applied to drive uptake Vehicle saline or PACAP (100 pmol) were concurrently applied for 30 minutes. A 100 μL media sample was taken for HPLC analysis. Clearance was quantified by subtracting the concentration of D-aspartate left in the media from the known

input concentration. Cells were then dissolved in 0.5% SDS and total protein for each well was quantified using the BCA method.

Glutamate/Aspartate HPLC: Glutamate and aspartate content was measured using fluorescence detection on an isocratic HPLC system (Shimadzu). A 10 µL sample underwent pre-column derivatization with orthophthaladldehdye (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. Peaks areas were quantitated in Shimadzu VP client software compared to known standards. Glutamate and aspartate content for each sample was normalized to total protein in the respective well.

Cocaine Self-Administration: Rats were trained to self-administer cocaine (0.5 mg/kg/inf, IV; 6 hr/day) or saline under a fixed ratio one schedule of reinforcement for 12 daily sessions. Rats then underwent a two-week abstinence period during which time they underwent stereotaxic surgery for cannula implantation targeting the NAcc (6^o, +1.2 mm anterior-posterior, +2.2 mm medial-lateral, -4.8 mm dorsal-ventral; injectors projected 2 mm past the end of the cannula). One week following recover, rats underwent extinction training sessions, which were identical to the self-administration sessions except lever presses resulted in saline infusions. Once rats met the extinction criterion of fewer than 15 presses/day, reinstatement testing was conducted. Reinstatement testing involved pretreatment with a high dose of cocaine (10 mg/kg, IP) in addition to a bilateral infusion of vehicle (saline or DMSO), $PACAP_{1-38}$ (100

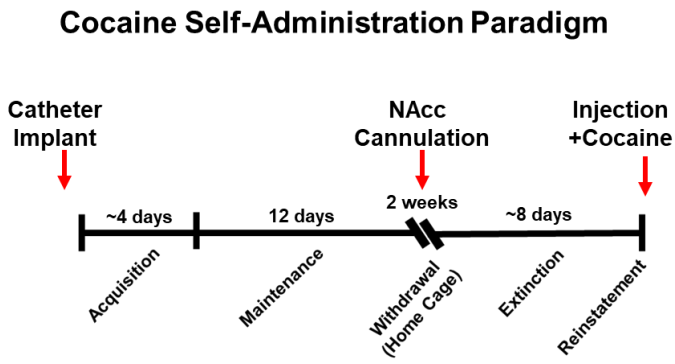

Figure 4-1: Schedule for long access cocaine self-administration paradigm.

pmol/side), RO-25-6981 (2 µg/side), SKF 81297 (0.125µl/min, 0.5µl total volume, 3 µg/side) ten minutes prior to testing and recording lever pressing for 2 hours.

Rhodamine Tracer injection: Wild type and Sxc rats were anesthetized with ketamine/xylazine and underwent stereotaxic surgery. Guide cannula (Plastics One) were implanted into the brain targeting the substantia nigra (0°, -5.0mm anterior-posterior, +2.1mm medial-lateral, -6.1mm dorsal-ventral) or ventral pallidum (0°, -0.6mm anterior-posterior, +2.8mm medial-lateral, -6.3mm dorsal-ventral). Micro injectors (Plastics One) were inserted into the cannula projecting +2.0mm to the target structure. 300 nL of rhodamine fluorescent latex microspheres (Lumafluor) were injected into the target region. Animals recovered for 2 weeks prior to use in experiments.

Electrophysiology: Whole-cell voltage-clamp recordings were made from medium spiny neurons (MSNs) in the core of the nucleus accumbens (NAc). MSNs retrogradely labeled with red beads that originated from either the SN or VP were selected for recording. NMDAR currents were induced by pressure ejection of NMDA and recorded at holding potential of -80 mV, -60 mV, -40 mV, -20 mV, 0 mV, 20 mV and 40 mV in the present of AMPAR antagonist CNQX (20

μM) and $GABA_A$ receptor blocker picrotoxin (100 μM). NMDA (25 μM) was pressure-ejected (5 psi, 300 ms) with a glass pipette (~1 μm tip opening). The I-V curve of NMDAR currents showed strong inward rectification due to Mg^{2+} (1.3 mM) blockade at negative holding potentials.

RESULTS

To investigate the role of PACAP signaling in the reinstatement of drug seeking, we employed a long-access cocaine self-administration paradigm. Once rats acquired, maintained, and extinguished their cocaine intake and seeking, they were subjected to a mock test. During the mock test, the rat was restrained to acclimate them to the microinjection procedure they would encounter during the reinstatement test. This was done to control for the stress of being restrained and microinjected. On the reinstatement test day, rats were given a bilateral infusion of vehicle (saline) or PACAP (100 pmol) into the NAcc paired with an injection of cocaine (I.P.,10 mg/kg). A two-way repeated measure ANOVA was used to determine differences in drug seeking (lever presses) during the cocaine-primed reinstatement test relative to a mock test (Figure 4-2 n=10/treatment). We report a main effect of PACAP treatment ($F_{1,18}$=13.03, p<0.05) and test procedure ($F_{1,18}$=45.89, p<0.05) with a significant interaction between these variables ($F_{1,18}$=13.82, p<0.05). Post-hoc tests revealed that 100 pmol PACAP treatment significantly reduced drug seeking on the reinstatement test day relative to 0 pmol (Tukey, p<0.05). These data demonstrate that PACAP attenuates cocaine-primed reinstatement of drug seeking when injected into the NAcc.

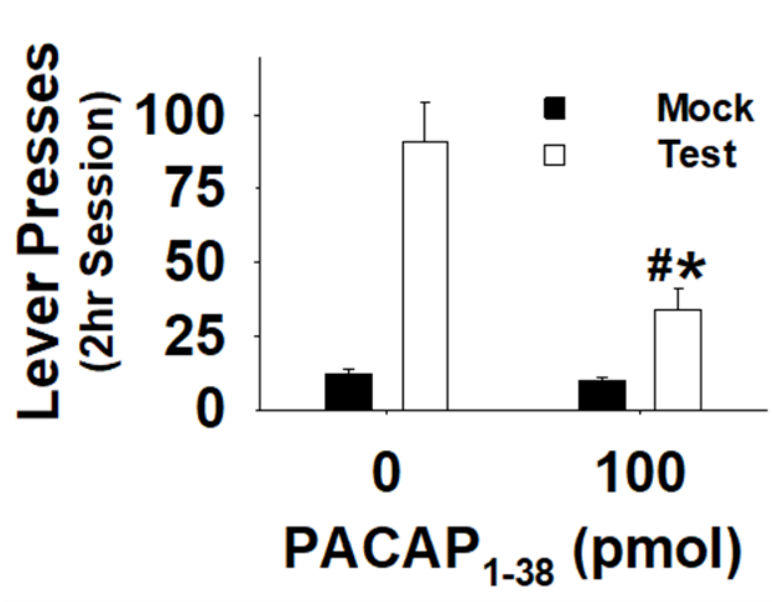

Figure 4-2: PACAP attenuates cocaine-primed reinstatement when infused into the NAcc. Data depicts number of lever presses during the reinstatement test relative to a mock injection test. Subjects were infused with PACAP or Saline bilaterally in the NAcc in conjunction with a 10 mg/kg cocaine injection I.P. *effect of PACAP treatment relative to vehicle $p<0.05$; # effect of PACAP treatment relative to mock $p<0.05$.

While PACAP attenuates drug seeking when exogenously applied to the NAcc, the endogenous expression of PACAP, its source, and receptor expression profile among astrocytes and neurons in the NAcc is unknown. To test if PACAP is endogenous to the NAcc, we sampled extracellular fluid using microdialysis and found PACAP to be present (Figure 4-3A). In order to ascertain the endogenous source of PACAP in the NAcc, the mPFC to NAcc pathway was labeled with a rhodamine retrograde tracer. This pathway in particular was chosen due to the relevance of the PFC in executive function and drug seeking (B. T. Chen et al., 2013). Fluorescent cells within the mPFC were extracted using fluorescent-activated cell sorting (FACS) and PACAP mRNA was detected in rhodamine positive but not rhodamine negative cells (Figure 4-3B). These findings demonstrate that PACAP is an endogenous peptide in the NAcc that is sourced by afferents from the mPFC.

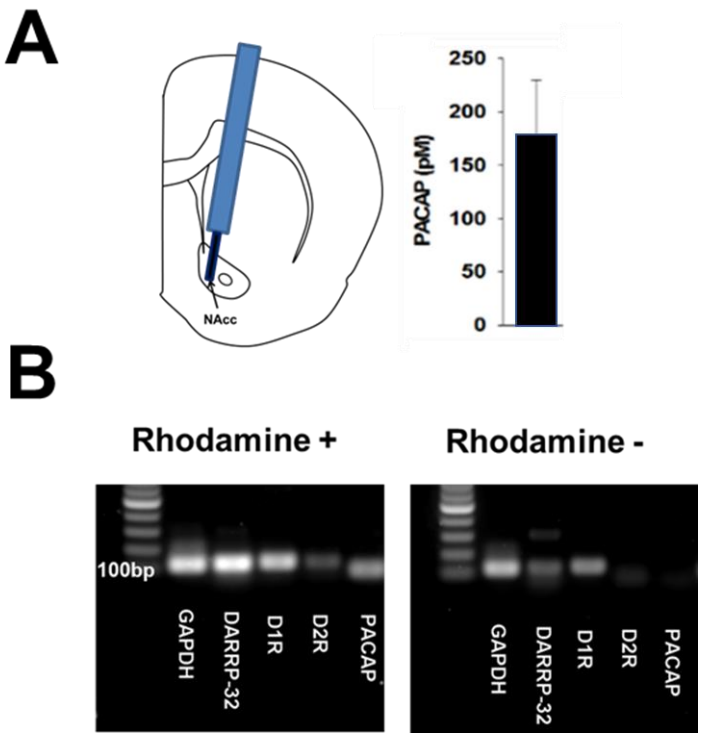

Figure 4-3. PACAP is present in the NAcc and is expressed in the mPFC to NAcc pathway. A, Diagram depicting microdialysis probe placement in the NAcc with PACAP concentration (pM). **B,** PCR products from FACS isolated rhodamine positive mPFC to NAcc neurons and rhodamine negative cell populations. D1R/D2R, dopamine 1 and 2 receptors.

Given the expression of PACAP in the NAcc we next investigated what the molecular effects of PACAP signaling in the NAcc are. Currently, the PACAP receptor expression profile and their functional impact in astrocytes and neurons within the NAcc are unknown. First, we isolated NAcc astrocytes from the GFAP: GFP-Lck transgenic rat via FACS so their PACAP receptor expression profile could be determined (Figure 4-4A). PCR products for xCT, PAC1R and VPAC2 were detected with GFAP and GAPDH as positive controls and NeuN as a negative control. Importantly, PACAP mRNA was not present in NAcc astrocytes which suggests they are not a source of PACAP.

Next, the effect of PACAP signaling on glutamate transporters in astrocytes was investigated. In the previous chapter we learned that PACAP (100 nM) increases Sxc-dependent cystine-evoked glutamate release from

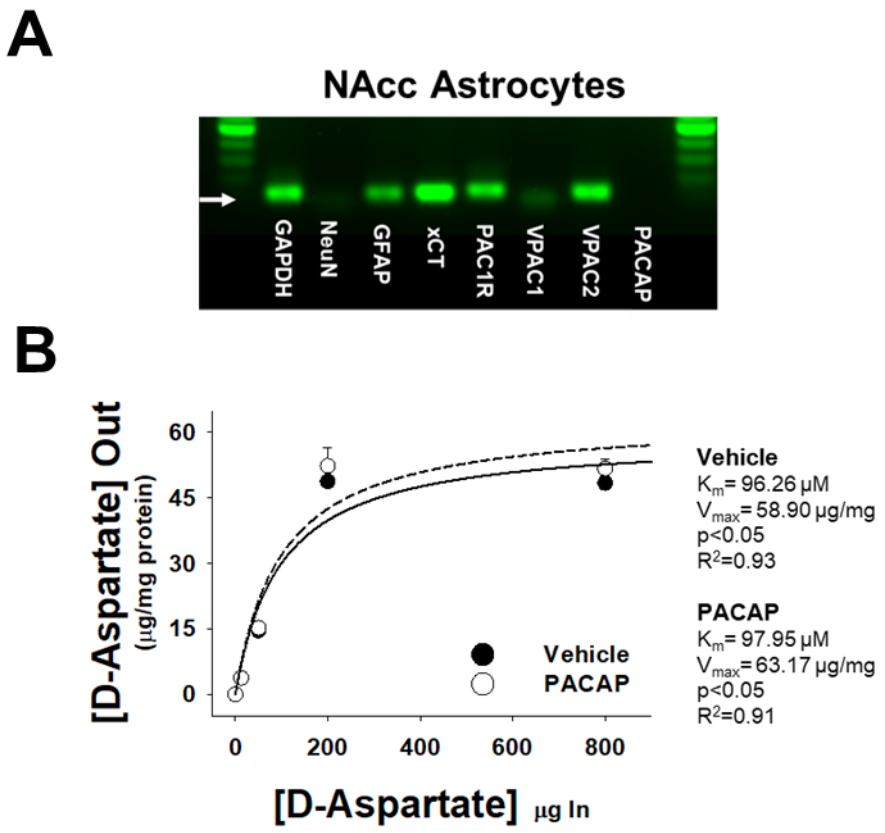

Figure 4-4: PACAP signaling in astrocytes does not impact D-aspartate uptake. A, PCR product from FACS isolated NAcc astrocytes. **B,** *In vitro* D-aspartate clearance with and without 100 pmol PACAP applied for 30 minutes. The Michaelis-Menten equation was used to estimate K_m and V_{max}.

striatal astrocytes. Here, we chose to investigate whether PACAP is influencing astrocytic EAAT function by measuring D-aspartate uptake (Figure 4-4B). The Michaelis-Menten equation was used to estimate changes in transport kinetics. This model equation strongly fit clearance in both vehicle and PACAP treatment groups (n=3-4/treatment, $p<0.05$) and explained 93% and 91% of the variability in the data, respectively. Relative to vehicle, PACAP produced marginal changes in D-aspartate transport kinetics (Km, 96.26 to 97.95 µM; Vmax, 58.90 to 63.17 µg/mg). Collectively, these data suggest that PACAP receptors are expressed by

astrocytes in the NAcc and PACAP signaling does not appear to rapidly impact on broad EAAT function.

Having established the presence of PACAP receptors in astrocytes and the effect they have on Sxc and EAATs, we next investigated the impact of PACAP signaling on NAcc efferents. First, we investigated PACAP receptor expression in the two major efferents from the NAcc: the direct pathway to the substantia nigra and the indirect pathway to the ventral pallidum. Both pathways were labeled with rhodamine tracer and single cells were extracted. PCR products for the dopamine receptors match established data (Kupchik et al., 2015) serving as a positive control, and PACAP receptors appear to be expressed in both pathways (Figure 4-5A,D). Intriguingly, the GluN2B subunit appears to only be expressed in the direct pathway.

Given the presence of PACAP receptors in both pathways, we next tested the physiological impact of PACAP signaling by measuring evoked EPSCs in labeled efferent pathways. In the direct pathway, PACAP (100 nM) significantly attenuated EPSC amplitude relative to baseline (Figure 4-5B; n=8; t_{13}=2.91, $p<0.05$) whereas in the indirect pathway, PACAP (100 nM) produced an increase relative to baseline (Figure 4-5E; n=8; t_{21}=2.59, $p<0.05$). Next, we investigated the contribution of NMDAR current to the pathway specific effects of PACAP signaling considering the expression of GluN2B is specific to the direct pathway. Intriguingly, PACAP enhanced NMDAR mediated current even at hyperpolarized holding potentials where Mg^{2+} block would otherwise prevent current conductance in the direct (Figure 4-5C; n=8-10/treatment; $p<0.05$) but not

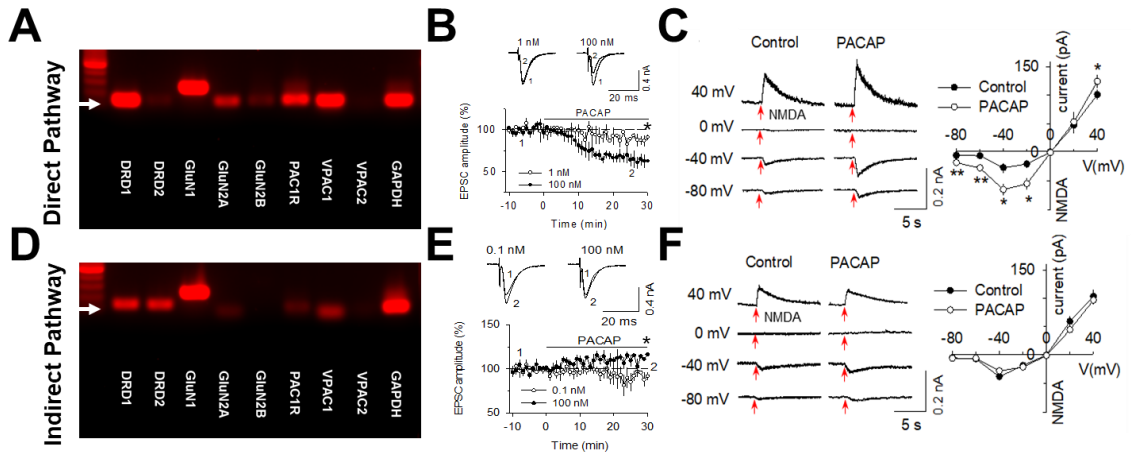

Figure 4-5: PACAP signaling in NAcc efferents differentially tunes excitatory output. **A,D,** PCR product from fluorescently labeled single cells from the direct and indirect pathway respectively. **B,E**, Data depicts evoked EPSCs from direct and indirect pathway MSNs, respectively, with and without PACAP treatment. **C,F,** IV curves depicting the effect of PACAP on NMDA mediated currents on direct and indirect pathway MSNs respectively. T-test, *p < 0.05; **p <0.01. Special thanks to Dr. Matt Hearing for collecting cells for A,D and Dr. Qing-Song Liu for B,C,E,F.

indirect pathway (Figure 4-5F, n=7-8/treatment, p>0.05). These data demonstrate that PACAP attenuates EPSCs but enhances NMDAR current independent of holding potential in the direct pathway, but enhances EPSCs in the indirect pathway while not impacting NMDAR currents.

Given that the effect of PACAP on EPSCs and NMDAR current is specific to the direct pathway, we next chose to investigate if PACAP is interacting with D1R or GluN2B containing NMDAR signaling since the expression profile of these two receptors appears to be the only difference among the two pathways. First, we determined if PACAP and D1R signaling are interacting to influence

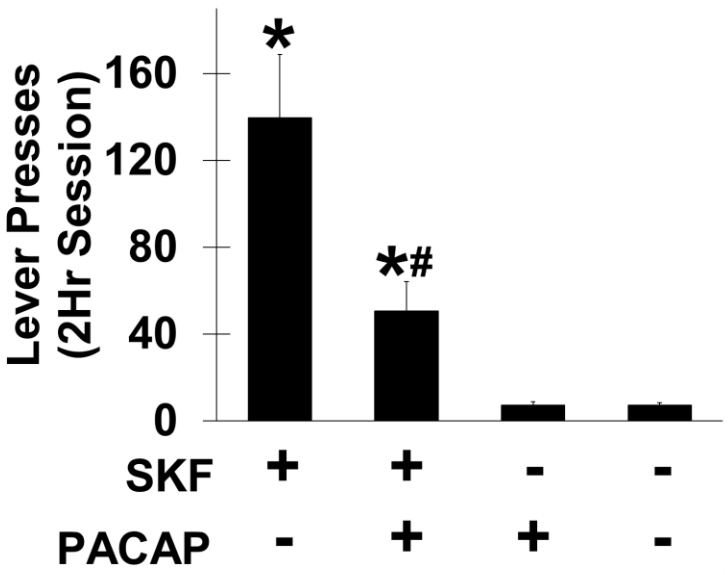

Figure 4-6: PACAP attenuates D1 receptor agonist-primed reinstatement. WT rats were infused with combinations of PACAP and SKF, a D1 receptor agonist during a reinstatement test. Cocaine was not administered. *effect of treatment relative to control p<0.05; #effect of PACAP relative to SKF p<0.05.

reinstatement of drug seeking. Following a long-access cocaine self-administration paradigm, WT rats were subjected to a microinjection of the D1R agonist SKF (2 μg/side), PACAP (100 pmol), or a combination of both into the NAcc (Figure 4-6). No cocaine was given during this reinstatement test. A one-way ANOVA revealed a significant effect of treatment ($F_{3,36}$=12.437; p<0.05). A Tukey test revealed a significant increase in drug seeking following SKF treatment relative to the vehicle (DMSO) control (p<0.05). When PACAP was co-administered with SKF, drug seeking was significantly attenuated relative to SKF alone (Tukey, p<0.05), but remained heightened relative to controls (Tukey, p>0.05). These data suggest that D1R agonism is sufficient to increase drug seeking which is partially blocked with the co-administration of PACAP which suggests that dopamine and PACAP signaling are interacting to influence the reinstatement of drug seeking.

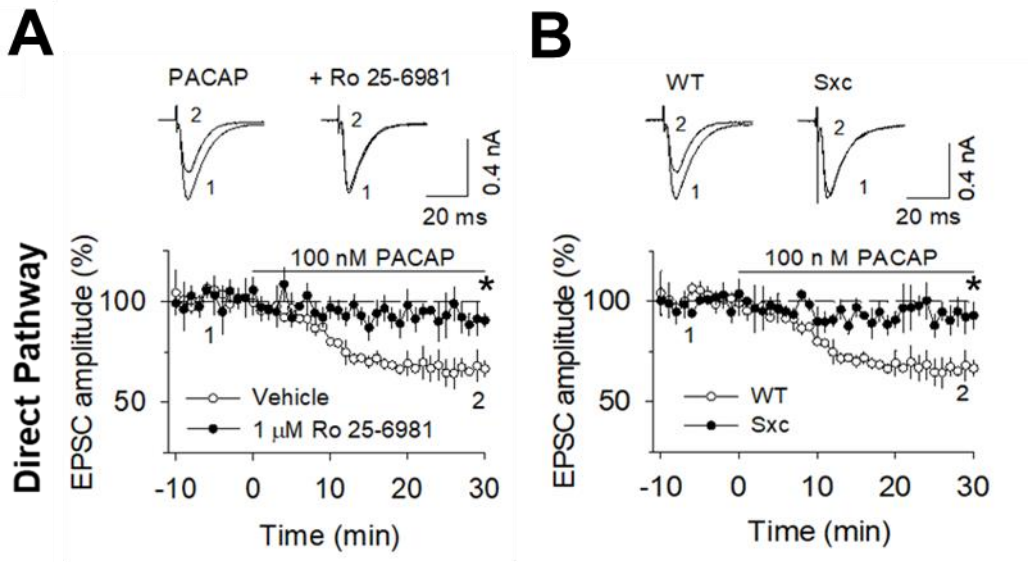

Figure 4-7: PACAP signaling requires Sxc and GluN2B to attenuate direct pathway evoked EPSCs. A, Data depicts evoked EPSC amplitude from direct pathway MSNs co-treated with PACAP ± RO-25-6981, a use-dependent GluN2B antagonist. **B,** Evoked EPSC amplitude from WT and MSxc rat direct pathway MSNs treated with and without PACAP (100 nM). T-test, *p<0.05. Special thanks to Dr. Qing-Song Liu for conducting these experiments.

The enhancement of NMDAR current while attenuating evoked EPSCs in the direct pathway is suggestive of a net inhibitory effect of NMDARs on EPSCs. Considering the pathway specificity of GluN2B we applied the GluN2B antagonist RO 25-6981 (RO) to direct pathway neurons in conjunction with PACAP, which revealed that GluN2B antagonism prevented PACAP from attenuating EPSCs (Figure 4-7A; n=7; t_{11}=2.97, p<0.05). Furthermore, the contribution of Sxc to this effect was also tested on slices from MSxc rats which revealed that PACAP could not attenuate EPSCs in the absence of Sxc (Figure 4-7B; n=7; t_{12}=3.09, p<0.01). These data reveal that PACAP attenuates evoked EPSCs and enhances NMDAR mediated current in the direct but not indirect pathway and these effects require GluN2B and Sxc.

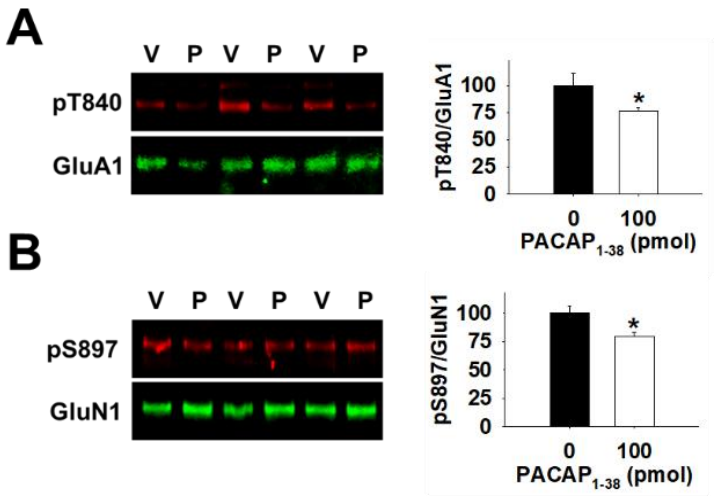

Figure 4-8: PACAP dephosphorylates GluA1 and GluN1 in the NAcc. A,B, Representative western blots from NAcc tissue samples from WT rats injected with V, vehicle or P, PACAP 100 pmol. Data depicts ratio of pT840/GluA1 and pS897/GluN1 fluorescence intensity. **p*<0.05 Student's T-test.

Given the attenuation of EPSCs in the direct pathway, we hypothesized that this may be due to a change in AMPAR and NMDAR phosphorylation in the NAcc. Two sites were investigated: T840 of GluA1 and S897 of GluN1. Given our previous data showing a link between T840 and Sxc as well as the aforementioned reduction by PACAP in hippocampal neurons (Toda & Huganir, 2015), we hypothesized that PACAP would decrease T840 in the NAcc. Indeed, a t-test revealed a significant reduction in the ratio of GluA1 phosphorylation at residue T840 to total GluA1 in rats microinjected with PACAP (100 pmol) into the NAcc relative to vehicle controls (Figure 4-8A; pT840/GluA1, n=7-8/treatment, t_{13} = 2.178; p<0.05).

Regarding S897 of GluN1, which is important for trafficking NMDARs to the synapse (B. Li et al., 2009), we hypothesized that PACAP signaling may be moving NMDARs away from the synapse, putting them in a position to receive Sxc-derived glutamate. A t-test revealed a significant reduction in the ratio of GluN1 phosphorylation at residue S897 to total GluN1 as a result of PACAP treatment (Figure 4-8B; pS897/GluN1, n=6-8/treatment, t_{12} = 2.386; p<0.05).

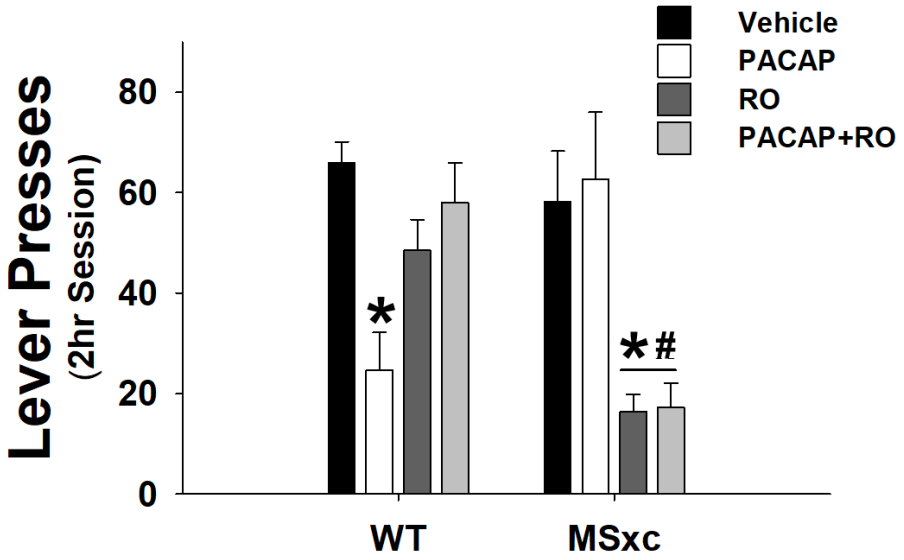

Figure 4-9: PACAP signaling requires Sxc and GluN2B to attenuate cocaine-primed reinstatement. WT and MSxc rats were infused with one of four treatments in conjunction with a 10 mg/kg cocaine injection I.P. *effect relative to vehicle $p<0.05$. #effect of treatment within genotype $p<0.05$.

These data suggest that PACAP influences GluA1 and GluN1 function in the NAcc by changing the phosphorylation of key residues that dictate the localization and or function of AMPARs and NMDARs. These biophysical changes correlate well with the finding that PACAP produces a net inhibition of EPSCs through a Sxc and GluN2B dependent mechanism.

Having demonstrated the necessity of GluN2B and Sxc for PACAP's ability to attenuate EPSCs in the direct pathway, we next tested their necessity in regulating drug seeking behavior. WT and MSxc rats were subjected to a long-access cocaine self-administration paradigm. During the reinstatement test, rats were given a cocaine injection in conjunction with infusion of vehicle control, PACAP, RO, or both into the NAcc (Figure 4-8). A two-way ANOVA revealed a main effect of treatment ($F_{3,52}=5.646$; $p<0.05$), no effect of genotype ($F_{1,52}=3.666$;

p>0.05), but a significant interaction between these variables ($F_{3,52}$=10.148; p<0.05). Post-hoc tests revealed PACAP attenuated reinstatement in WT (Tukey, p<0.05) but not MSxc rats (Tukey, p>0.05) relative to vehicle. RO did not significantly impact cocaine seeking in WT rats (Tukey, p>0.05), but did prevent the attenuation of seeking when co-administered with PACAP (Tukey, p<0.05, relative to PACAP alone; p>0.05, relative to vehicle). In MSxc rats, RO attenuated drug seeking (Tukey, p<0.05, relative to vehicle) even in the presence of PACAP (Tukey, p<0.05, relative to PACAP alone). These data suggest that the effects of PACAP on cocaine seeking require Sxc and GluN2B, but there are genotypic differences in the effect of RO.

DISCUSSION

The purpose of these experiments was to investigate how neuronal and astrocyte signals are coordinated in the NAcc to alter network output and impact cognitive control in drug seeking. These data demonstrate that the neuropeptide PACAP, which is expressed by the mPFC to NAcc afferent, is able to attenuate drug seeking via a multi-cell signaling mechanism that coordinates glutamate release from astrocytes via Sxc and ligand binding of this signal in neurons by GluN2B containing NMDARs. Remarkably, PACAP can precisely enhance NMDAR current in the direct but not indirect pathway which results in a net inhibition of evoked EPSCs. This phenomenon mimics a long-term depression-like mechanism with the key features of astrocytic glutamate recruitment and biophysical adaptation of neuronal NMDARs which collectively dephosphorylate GluA1 and dampen excitatory output (Figure 4-10). In turn, the dampening of the

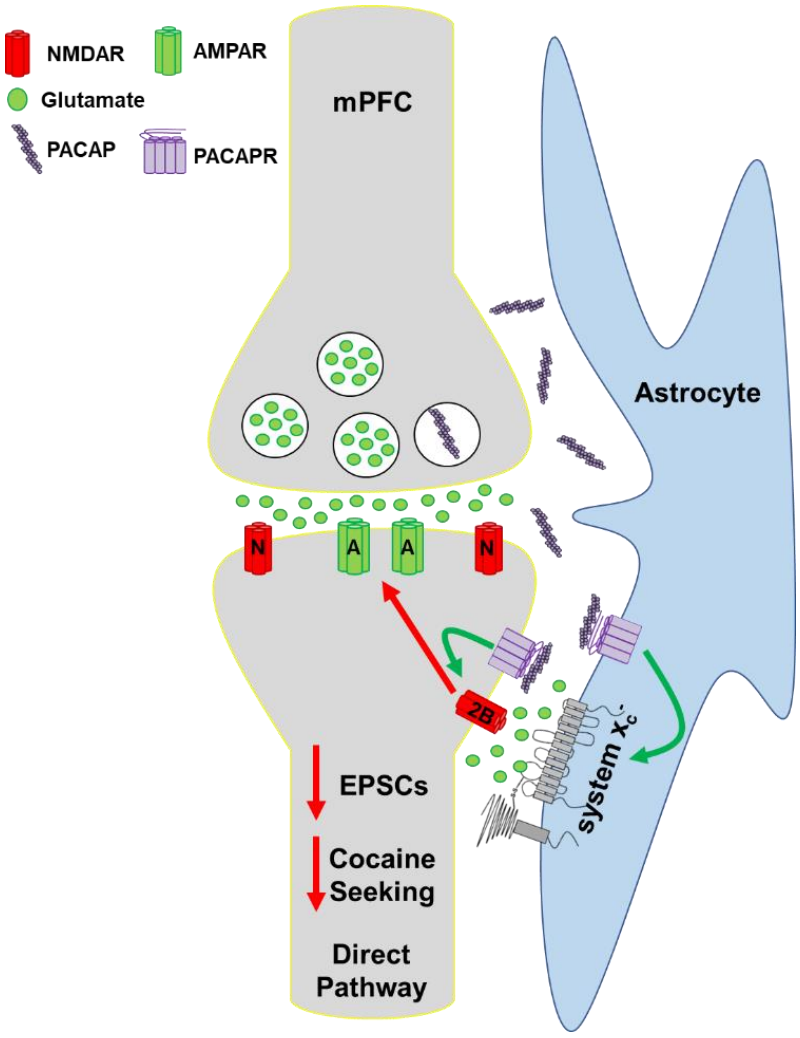

Figure 4-10: Multi-cell signaling mechanism for PACAP signaling in the NAcc. PACAP sourced from the mPFC signals through astrocytes to enhance Sxc function while also signaling through neurons to localize NMDA receptors to the extrasynaptic compartment to receive astrocytic glutamate. This produces an inhibitory effect on GluA1 function in the direct pathway which attenuates cocaine-primed reinstatement.

direct pathway corresponds to reduced cocaine-primed reinstatement of drug seeking.

The effect of acute PACAP application to astrocytes demonstrates rapid increases in Sxc-mediated cystine-evoked glutamate release with no changes to broad EAAT function as measured by D-aspartate uptake. Previous studies had shown that the expression and function of Sxc, measured by cystine uptake, is increased following 24-hour or greater application of PACAP (Kong et al., 2016; Resch et al., 2014). Similarly, GLT-1 expression and function were upregulated by PACAP following a 72-hour incubation (Figiel & Engele, 2000). Given the

timescale of these changes, it is unlikely that these effects would be responsible for the rapid behavioral effects of PACAP. Thus, more rapid assays consisting of 30-minute PACAP treatment were conducted. While not much is known about post-translational modifications to xCT that produce changes in Sxc-dependent glutamate release, PACAP signaling may produce a change in phosphorylation status at one or more of the three putative regulatory sites S26, S185, or S481 to enhance transport efficiency. Furthermore, the lack of changes in EAAT function at 30 minutes suggests this specific effect may be restricted to long-term effects of PACAP signaling. Collectively, the principle effect of PACAP signaling in astrocytes is enhancement of glutamate release via Sxc.

The impact of PACAP signaling on the direct pathway is significant given the established roles of striatal efferents in reward seeking. When D1R positive neurons are stimulated optogenetically, cocaine-conditioned place preference and cocaine induced locomotor activity is enhanced, with the opposite effect occurring when D2R positive neurons are stimulated (Lobo et al., 2010). A major unanswered question is how PACAP can selectively regulate the direct pathway, given its receptors appear in both efferents. Preliminary evidence suggests that the GluN2B subunit is specifically expressed by the direct pathway, which may explain why this pathway is regulated by Sxc. Another major difference between the two pathways according to our data is the expression of the dopamine receptors D1R and D2R with the direct pathway exclusively expressing D1R and the indirect pathway expressing a mix of D1R and D2R. Importantly, D1Rs have been shown to enhance AMPAR mediated signaling through increased trafficking

to the membrane (Chao, Ariano, Peterson, & Wolf, 2002; Gao, Sun, & Wolf, 2006). Since PACAP has a net inhibitory effect on AMPAR signaling, we were curious about the potential interaction between PACAP and dopamine signaling on cocaine seeking. Our data revealed that PACAP and D1R signaling interact to regulate drug seeking with PACAP attenuating but not blocking D1R agonist induced reinstatement.

Mechanistically, it is likely that PACAP and dopamine are interacting at the AMPAR to gate excitatory output in the direct pathway. In support, application of SKF to NAcc neurons increases GluA1 insertion into the membrane (Chao et al., 2002). A follow up study, albeit in hippocampal neurons, demonstrated that GluA1 was preferentially inserted into the extrasynaptic compartment and subsequently laterally diffused into the synapse following NMDAR stimulation via glycine (Gao et al., 2006). Within the context of our findings, this mechanism is significant because the initial insertion of GluA1 into the extrasynaptic compartment puts them in optimal position to be regulated by extrasynaptic NMDAR. If the two-cell PACAP signaling mechanism is engaged, the newly inserted D1R-dependent GluA1 would be dephosphorylated at T840 and thus functionally dampened prior to their lateral diffusion into the synapse. In this way, PACAP receptors and D1Rs are competing for control over AMPAR function and ultimately control over direct pathway MSNs and reward seeking behavior. Certainly, more research is required to understand the electrophysiological and molecular factors that may be influencing this mechanism.

The expression of PACAP in the PFC afferent to the NAcc is significant finding for the addiction field and may explain why Sxc function in the NAcc is reduced following drug intake. Disruption of the mPFC to NAcc pathway has been suggested to be critical for the reinstatement of cocaine seeking. In particular, long-term cocaine self-administration can broadly reduce the excitability of PFC pyramidal neurons and when these neurons are optogenetically stimulated, cocaine seeking is reduced (B. T. Chen et al., 2013). One interpretation in line with our results is that neurons containing PACAP projecting to the NAcc are being activated which is producing the reduction in seeking. It could be that over the course of drug intake, the PFC is taken off-line and PACAP is no longer being released into the NAcc from this afferent, which causes Sxc function to decrease. By infusing PACAP into the NAcc during a reinstatement test, we are rectifying this reduction. However, this does not rule out the possibility that other glutamatergic NAcc afferents such as from the basolateral amygdala which are also critical for cocaine seeking (B. R. Lee et al., 2013) may contain PACAP. Future research should be aimed at determining whether dysregulation in PACAP production or release from NAcc afferents is predictive of the propensity for relapse.

While speculative, PACAP may encode qualitative information about incoming stimuli; signaling to local astrocytes that the incoming stimulus information is strong and requires astrocytes to help in processing and disseminating this information to the network. Importantly, peptides are typically released from neurons under different circumstances relative to small

neurotransmitters, including specific patterns of firing or long durations of activity (Cropper, Jing, Vilim, & Weiss, 2018). Thus, the information coming from the mPFC or possibly other NAcc afferents is likely specific to the environmental circumstances and PACAP is released to signal to astrocytes that they are needed to process this information. Given the actions of PACAP, it may encode negative feedback about the probability of a rewarding outcome, which may be why it potently blocks reinstatement.

The biophysical adaptations of GluA1 and GluN1 produced by PACAP are consistent with and expand on previously established findings. PACAP signaling in hippocampal neurons results in a reduction in pT840/GluA1. Importantly, this phenomenon requires NMDAR activation (Toda & Huganir, 2015). We found that PACAP in the NAcc can produce the same reduction in pT840 which coincided with enhancement of NMDAR currents and an attenuation of evoked EPSCs in the direct but not indirect pathway. Intriguingly, PACAP was able to enhance NMDAR currents even at holding potentials where Mg^{2+} block would otherwise be present. One interpretation of this is that PACAP is altering NMDAR to make them resistant to Mg^{2+} allowing them to be more rapid in their signal transduction capabilities rather than coincidence detectors that require sufficient antecedent AMPAR mediated depolarization. In support of this theory, protein kinase C (PKC) reduces Mg^{2+} block of NMDARs (L. Chen & Huang, 1992) and PACAP activates PKC (Macdonald et al., 2005). The reduction in Mg^{2+} block allows any neuron in the network that contains these modified NMDARs to be disengaged in a synchronized fashion independent of activity state, which could be argued as

an incredibly powerful and efficient inhibitory signal. Despite this, a lingering question remains; how is PACAP able to do this in a pathway specific fashion even though its receptors appear to be expressed by both efferents?

One interpretation of the pathway specific effects of PACAP is that only the astrocytes that are interacting with the direct pathway contain Sxc and therefore PACAP and Sxc can only effectively alter that network. On the other hand, the NMDAR encoding Sxc-derived glutamate may only be expressed by the direct pathway. Preliminary evidence suggests that only the direct pathway contains the GluN2B subunit. This is a controversial claim, which will require more research to fully investigate, but the findings that GluN2B is necessary for the effects of PACAP on EPSC amplitude in the direct pathway and consequently drug seeking behavior support this interpretation. Furthermore, there was evidence that PACAP enhances EPSCs in the indirect pathway. It is not clear whether Sxc is involved in this regulation. The enhancement of the indirect pathway could be due to PACAP signaling through its receptors on these neurons to increase phosphorylation of residue S845 of GluA1 which has been shown to enhance the synaptic retention and ion conductance of AMPARs (Diering, Heo, Hussain, Liu, & Huganir, 2016; Toda & Huganir, 2015). If GluN2B is not expressed by the indirect pathway, T840 of GluA1 is unlikely to be impacted which places AMPARs in an enhanced rather than inhibited state following PACAP treatment.

The finding that pS897/GluN1 is dephosphorylated because of PACAP signaling is novel and suggests that neural signals can control glutamate

receptor localization. When pS897/GluN1 is mutated into a phospho-resistant residue, the expression of GluN1 and GluA1 significantly decreases in hippocampal synapses which coincides with an impairment in LTP induction (B. Li et al., 2009). Since PACAP is dephosphorylating pS897/GluN1, it should reduce synaptic localization of GluN1. It is unclear if these receptors are internalized or lateralized into the extrasynaptic compartment, but in either scenario, extrasynaptic NMDAR would likely be dominant. Given that pT840/GluA1 is a pro-synaptic enrichment marker regulated by NMDAR activation, the net outcome of this extrasynaptic NMDAR signal is likely dephosphorylation of pT840/GluA1 which produces an LTD-like effect. Given PACAP's actions on NMDAR mediated and evoked EPSCs, it is likely destabilizing synaptic glutamate receptors in a pathway specific manner thus reducing their ability to encode synaptic glutamate.

The ability of astrocytes to potentiate and depress neural networks is emerging as a fundamental principle of brain function (Martin et al., 2015). Initial investigations into this phenomenon revealed the role of D-serine release from astrocytes, an NMDAR co-agonist, in the potentiation of synapses in the hippocampus (Henneberger, Papouin, Oliet, & Rusakov, 2010). This release mechanism has been demonstrated to be sensitive to intracellular Ca^{2+} and dependent on vesicular fusion. However, the causative factors or receptors that drive this mechanism remain unclear, but theoretically any factor that can produce broad changes in Ca^{2+} within an astrocyte could be involved. PACAP signaling through its Gq coupled receptors could theoretically trigger Ca^{2+}

transients in astrocytes, but other factors we previously explored such as endocannabinoids could also engage this mechanism. Future experiments should be aimed at discerning the intracellular signaling cascades triggered by PACAP signaling and whether they are sufficient to induce release of non-glutamatergic gliotransmitters that modify neural networks.

The reinstatement data from WT and MSxc rats reveals how GluN2B can have different effects on cocaine seeking depending on Sxc activity. In WT rats treated with cocaine and RO, which represents basal Sxc activity, there was no significant effect on cocaine seeking relative to vehicle. With a PACAP treatment, which represents enhanced Sxc function, cocaine seeking is reduced, but if RO is co-administered, this reduction is blocked. Conversely, in MSxc rats, which represents an absence of Sxc activity, cocaine with RO alone is sufficient to attenuate cocaine seeking and PACAP is unable to alter cocaine seeking with RO present. This suggests the following: when Sxc activity is unaltered, GluN2B has minimal impact on cocaine seeking. When Sxc activity is enhanced, GluN2B is involved in attenuating cocaine seeking and when Sxc activity is absent, GluN2B drives drug seeking. From a therapeutic perspective, this highlights the difficulty in targeting receptors to treat disorders; the efficacy and behavioral outcome of the drug may be different depending on, in this instance, the status of glutamate release from astrocytes. Given the multitude of biological variables that could impact drug efficacy such as time of day, bioavailability, and sex, it is not surprising that unveiling effective therapies for addiction relapse has been difficult.

Collectively, these data demonstrate that PACAP signals through astrocytes and neurons to coordinate astrocytic release and neuronal reception of glutamate. This dual-cell signaling mechanism attenuates excitatory output in the direct but not indirect pathway and attenuates cocaine-primed reinstatement of drug seeking which requires Sxc and GluN2B. It is possible that PACAP is encoding stimulus information from the mPFC that signals to astrocytes that they are needed to help process this information and actualize it into a functional output through regulating specific NAcc efferents. From a therapeutic perspective, PACAP has revealed the significance of Sxc and GluN2B in regulating drug seeking behavior. Given the emerging literature on Sxc-targeting compounds and the recent approval of NMDAR targeting drugs such as Esketamine for severe depression by the FDA (Gould, Zarate, & Thompson, 2019), it is possible that we have begun to unveil novel therapeutic targets as a result of an increased understanding of intercellular communication involving astrocytes. However, our behavioral data reveals that more research will need to be done to understand how manipulating GluN2B signaling can have varying outcomes on cocaine seeking. Ultimately, these findings might have therapeutic implications for not only substance abuse, but other neuropsychiatric diseases.

CHAPTER V:
PERSPECTIVES ON THE IMPORTANCE OF SYSTEM XC- TO INTERCELLULAR COMMUNICATION AND COGNITIVE PROCESSING

The goal of these studies was to investigate the novel idea that the evolutionary expansion of signaling complexity between brain cells contributed to evolutionary gains in cognitive ability. Progress in this area has the potential to transform efforts to treat cognitive dysfunction that is present in an array of CNS disorders. The primary benefit of the findings reported here was to illustrate the relevance of signaling mechanisms in astrocytes, which are among the most abundant cells in the human brain. Specifically, we provide novel support for the hypothesis that evolutionarily-new glutamate signaling mechanisms expressed by astrocytes are selectively required for complex forms of cognition.

Our study of astrocyte signaling focused on the characterization of the cysteine-glutamate antiporter Sxc. We were interested in studying Sxc function from the molecular to the behavioral level. As discussed below, our data provides preliminary insights into how Sxc has been integrated into neuronal and tripartite signaling mechanisms (Figure 5-1) to produce complex regulation of behavioral control. However, the ultimate significance of these studies will be determined by the future lines of research triggered by our findings.

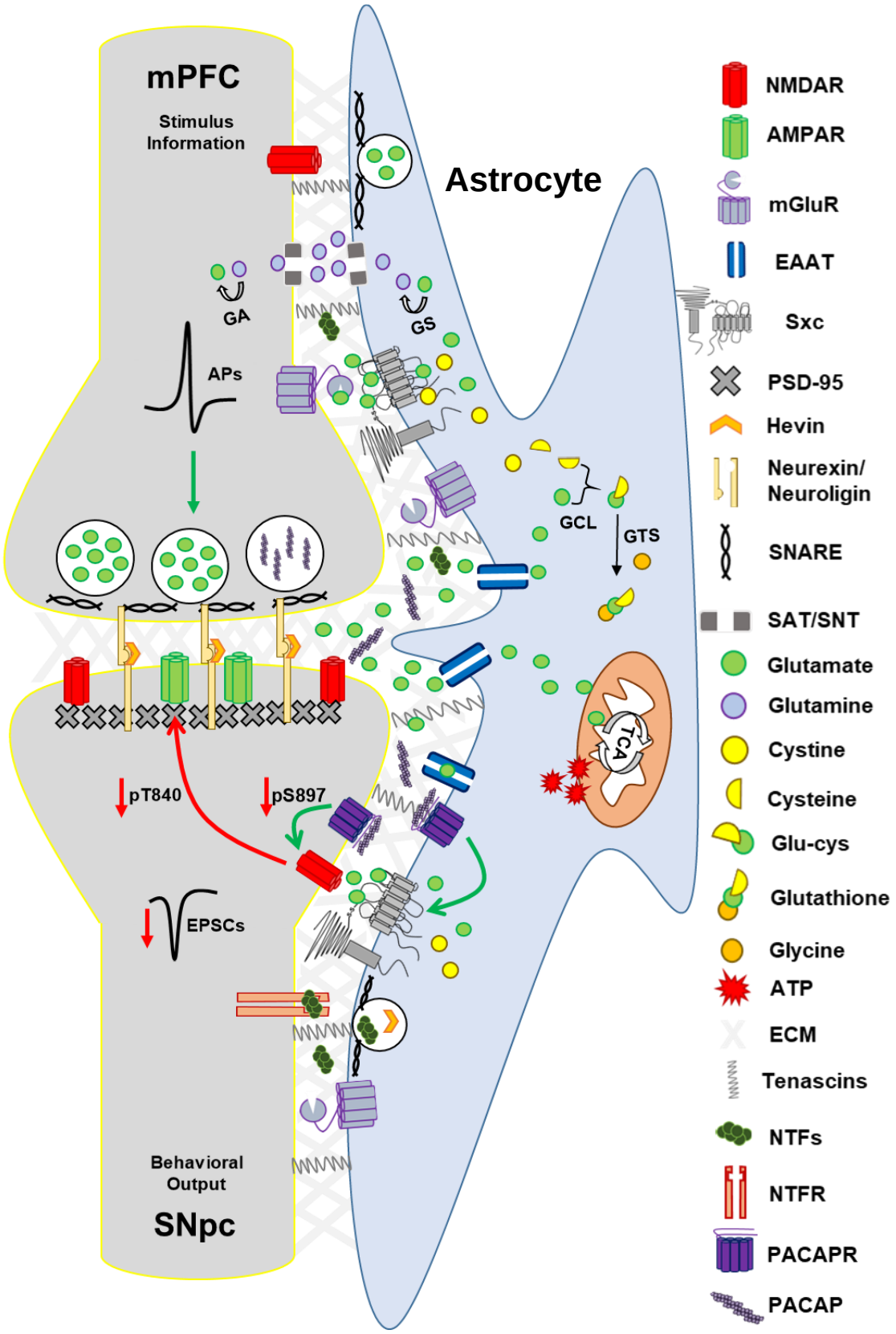
mPFC
Stimulus Information
Astrocyte
GA
GS
APs
GCL
GTS
TCA
pT840
pS897
EPSCs
Behavioral Output
SNpc
NMDAR
AMPAR
mGluR
EAAT
Sxc
PSD-95
Hevin
Neurexin/ Neuroligin
SNARE
SAT/SNT
Glutamate
Glutamine
Cystine
Cysteine
Glu-cys
Glutathione
Glycine
ATP
ECM
Tenascins
NTFs
NTFR
PACAPR
PACAP

Figure 5-1: How PACAP and Sxc fit into the model of tripartite synapse function. The major advancements of this work are highlighted by the inclusion of PACAP, its receptors, and the net outcome of the intercellular communication produced by its signaling. PACAP is released by afferents from the mPFC, and possibly other afferents and is received by receptors expressed by neurons and astrocytes. Within astrocytes, PACAP enhances Sxc-mediated glutamate release. In neurons, PACAP likely facilitates dephosphorylation of GluN1 at S897 which concentrates NMDARs in the extrasynaptic compartment. Here, GluN2B containing NMDARs encode glutamate released by Sxc and display enhanced conductance even during hyperpolarized potentials. The significance of this phenomenon is that antecedent depolarization via AMPARs is not required to remove Mg^{2+} block from the NMDAR which allows Sxc to activate the NMDAR of silent neurons. Activation of GluN2B inhibits evoked EPSCs, likely through the dephosphorylation of GluA1 at T840. This cellular and molecular mechanism specifically attenuates the direct pathway from the NAcc to the substantia nigra pars compacta (SNpc) which effectively gates the ability for this pathway to elicit a behavioral response.

The Significance of Sxc to the Tripartite

The significance of glutamate release from astrocytes to signaling within the tripartite is evidenced by our findings that Sxc in the NAcc influences neuronal (e.g., ionotropic glutamate receptors) and astrocytic (glutamate transporters) signaling mechanisms, and that it in turn is regulated by multiple neuronal mechanisms (e.g. PACAP, endocannabinoids). In support, a loss of Sxc impaired astrocytic EAAT function, which would likely produce an increase in synaptic (and extrasynaptic) concentrations of extracellular glutamate. Additionally, a loss of Sxc does not appear to impact presynaptic release of glutamate, which was surprising given a previously established link between the two (Baker et al., 2002). However, Sxc activity was found to contribute to the tuning of glutamate signaling within the synapse by negatively regulating AMPAR function, an effect that we and others indicate is likely mediated by extrasynaptic NMDARs (Delgado et al., 2007). Regulation of AMPAR function likely involves the regulation the phosphorylation of GluA1, specifically residue GluA1 T840,

that has been shown to influence AMPAR clustering in the synapse (Williams & Featherstone, 2014). This is an important finding because the regulation of synaptic transmission by numerous signaling mechanisms, including dopamine transmission (Chao et al., 2002; Gao et al., 2006), may involve the regulation of AMPARs. Hence, these receptors appear to be a site of convergence for highly disparate signaling mechanisms within the tripartite.

Much of what we learned about the molecular and cellular roles for Sxc involved its actions in the NAcc. We found that Sxc contributes to the gating of NAcc efferent pathways in a manner that regulates behavior. In particular, a surprising finding was the apparent involvement of Sxc in suppressing the activity of the direct pathway (NAcc-SN) although there was not a clear effect of loss of Sxc on the second major efferent, the indirect pathway (NACC-VP). Given that the direct pathway is canonically viewed to drive motivated behavior (Bromberg-Martin et al., 2010; Lobo et al., 2010), the regulation of this pathway by Sxc suggests that astrocytes contribute to local processing of incoming information to modify behavioral output. Presumably, Sxc activity in other structures, such as the prefrontal cortex, also functions to provide complex, potentially pathway-specific gating of afferent and/or efferent outputs in a manner that facilitates cognition.

A critical issue we examined was how Sxc-dependent astrocyte to neuron signaling is regulated. While astrocytes are increasingly implicated in the regulation of neural circuits, there is a need to understand how glutamate release from astrocytes is coordinated with neuron to neuron signals. First, we examined

the regulation of Sxc by mechanisms often linked to the control of neuron-neuron signaling by determining the capacity of corticosterone, endocannabinoids, and PACAP to produce rapid changes in Sxc activity. In part, these mechanisms were selected because they reflect control provided by presynaptic neurons, postsynaptic neurons, and circulating hormones. We found each of these signaling mechanisms to exert rapid regulation of Sxc activity. The ability for these factors to influence Sxc suggests that even though Sxc is astrocytic and evolutionarily-new, it has become integrated into the tripartite.

An exciting question that remains to be answered is at what point on the evolutionary timescale did neural and hormonal factors develop the ability to influence Sxc function. To address this question, we searched for putative consensus phosphorylation sequences on xCT, and then mapped their appearance on the evolution of vertebrates to find three conserved regulatory sites, S26, S185, and S481. S481 is interesting considering this site exists in mammals but no other vertebrates. In contrast, S26 is only expressed in some birds and mammals. Because these serine residues are targeted by MSK1, which is downstream of glucocorticoid, PACAP, and MAP kinase signaling (Beck et al., 2013; Butcher et al., 2005), an important question that remains is how PACAP and corticosterone signaling interact to influence the phosphorylation of these residues and consequently change Sxc function. Furthermore, why certain organisms have these regulatory sites versus others needs to be answered as it will help illuminate their biological significance. Given these findings, Sxc has

been evolving in vertebrates to engrain itself into the processing of information provided by neurons and the hormonal milieu.

The Role of PACAP in Shaping Sxc-Mediated Astrocyte to Neuron Signaling

The regulation of Sxc by PACAP was especially intriguing to us largely due to the profound effects that this neuropeptide has been shown to produce on glutamate transporters and receptors (Figiel & Engele, 2000; Kong et al., 2016; Resch et al., 2014; Toda & Huganir, 2015). As a result, we chose to investigate this Sxc-regulatory mechanism in greater detail. We found PACAP to be expressed by mPFC-NAcc neurons and is present in the interstitial space of the NAcc. Collectively, these are the first data to implicate PACAP as a neuropeptide in NAc afferents. The distribution of PACAP receptors provided interesting insights into its function. We found that PACAP appears to enable, and possibly control the impact, of Sxc-mediated astrocyte to neuron signaling by acting on both astrocytes and potentially receptive neurons.

PACAP receptors were detected in NAcc astrocytes, which likely explains how this neuropeptide is able to exert rapid regulation of cystine-evoked glutamate release by Sxc. To our surprise, the capacity of PACAP to regulate astrocytic glutamate mechanisms did not extend to rapid control of EAAT function as measured by D-aspartate uptake. However, this does not rule out the possibility that PACAP may engage glutamate transporters other than Sxc and EAATs to regulate extracellular glutamate.

Neuronal PACAP receptors may define the nature or impact of incoming signals from astrocytes. First, we learned that PACAP dephosphorylates GluN1

at residue S897. This is interesting because this site has been shown to promote diffusion of the NMDA receptor out of the synapse (B. Li et al., 2009), where it may be exposed to Sxc-released glutamate. Second, neuronal PACAP signaling appears to relieve NMDAR blockade by Mg^{2+}. This unexpected finding may add to a limited body of work indicating that Mg^{2+} blockade is a powerful form of NMDAR regulation by intracellular signaling pathways (L. Chen & Huang, 1992; Hahn, Wang, & Margeta, 2015; Moriguchi et al., 2007; Tyszkiewicz, Gu, Wang, Cai, & Yan, 2004). In the absence of PACAP-induced removal of Mg^{2+} blockade, Sxc-activation is unlikely to stimulate extrasynaptic NMDA receptors. Instead, Sxc regulation of neuronal function is traditionally linked to metabotropic receptors (M. M. Moran et al., 2005; Moussawi et al., 2011). Hence, the question becomes, how might activation of extrasynaptic NMDARs produce unique changes to neuronal activity? Extrasynaptic NMDARs have been shown to inhibit synaptic transmission by altering the phosphorylation of multiple residues of the AMPAR subunit GluA1, (Delgado et al., 2007; Toda & Huganir, 2015). Consistent with this, we observed PACAP-induced suppression of synaptic transmission, which was dependent on both Sxc and GluN2B-expressing NMDARs.

The attenuation of synaptic activity by PACAP-mediated Sxc to NMDA signaling was pathway specific, which raises the possibility that PACAP also determines which cells are impacted by Sxc-mediated astrocyte to neuron signaling. In support, we observed PACAP-induced regulation of NMDA receptor function and synaptic activity in the direct but not the indirect pathway. Similarly, we observed pathway-specific differences in expression of the various PACAP

receptors and in the expression of GluN2B. While these preliminary observations require further study, these findings provide a potential mechanism illustrating how astrocytes can produce cell- and pathway-specific regulation of synaptic activity even when maintaining tonic glutamate concentrations throughout the diffuse, interstitial space.

In addition to discrete signaling mechanisms such as PACAP, the complexity of the tripartite may contain forces regulating glutamate receptor activation. For example, we have long assumed that extrasynaptic, interstitial concentrations of glutamate were relatively homogenous, and therefore displayed a volume transmitter type of signaling. This information has been used to form predictions about extracellular glutamate concentrations, receptor affinity for glutamate, and patterns of receptor activation. To the extent that we considered areas of extracellular glutamate to display spatial-dependent differences in concentration, the assumption was that equilibrium in glutamate concentration was solely due to a diffusion pattern determined by concentration gradients. However, modern findings in neuroscience indicate that it may time to reexamine what we think we know.

Is Sxc-NMDAR Signaling Impacted by the Structure of the Extrasynaptic Space?

Is glutamate movement in the interstitial space solely determined by glutamate concentration? This is often assumed to be the case in the extrasynaptic space. Specifically, we have long assumed that astrocytes maintain a near homogenous concentration of glutamate that reflects the relative affinity of glutamate receptors. Theoretically, a much more efficient and effective

mode of intercellular signaling would be a system in which the release site of glutamate was targeted toward the receptive site (e.g., the receptor populations) akin to the synapse. In the context of our findings, the question is whether PACAP enables a very specific signaling axis between astrocytes and neurons by the precise targeting of NMDARs. A critical advantage of this system would be the capacity to create multiple, functionally distinct, signaling microdomains that could be more efficiently maintained due to the reduced need of extracellular glutamate molecules. The reduction in the number of glutamate molecules would be due to the reduction in the extracellular space requiring a set concentration of glutamate that would be capable of activating the receptor.

The extracellular matrix, which represents the structural foundation of neural circuits, is gaining recognition for its role in compartmentalizing the brain to create the types of signaling efficiencies described above (Dityatev et al., 2010). An important question is whether the extracellular matrix physically links astrocytic Sxc with neuronal glutamate receptors that are trafficked into the appropriate zone in a cell- and signal- specific manner. Again, this would mimic vesicular release machinery, which adheres to adjacent post-synaptic density proteins (Christopherson et al., 2005; Eroglu et al., 2009; Hata et al., 1993; Kania & Klein, 2016; Kucukdereli et al., 2011).

Assessing the likelihood that Sxc interacts with the extracellular matrix and possibly other transmembrane cellular adhesion proteins on neurons to localize to adjacent to NMDARs requires insight into the crystalline structure of the Sxc subunits. Importantly, there are two subunits of Sxc, the light chain xCT and

heavy chain 4F2hc (or designated as CD98), which is also essential for the expression of the light chain in the membrane. CD98 has many light chain partners (Mastroberardino et al., 1998) interacting across three domains: cytoplasmic, transmembrane, and extracellular all with their unique functions. The cytoplasmic and transmembrane domains of CD98 interact with cell adhesion proteins such as integrins to facilitate their binding to their extracellular ligands (Fenczik et al., 2001). Integrins have a number of different extracellular ligands such as components of the basement membrane of the extracellular matrix (collagens, tenascins, laminins etc.) and can also facilitate direct cell-cell interactions (Humphries, Byron, & Humphries, 2006). The extracellular domain of CD98 is important for facilitating amino acid transport through the light chain, i.e. xCT, due to the covalent interaction of cysteines within this domain with the light chain (Fenczik et al., 2001). Furthermore, CD98 has been well studied by cancer researchers due to its ability to interact with cellular adhesion proteins such as integrins, which facilitate the migration of cancer cells through the extracellular parenchyma (Feral et al., 2005). However, this phenomenon is not limited to cancer cells, as a similar phenomenon is observed in epithelial cells (Cai et al., 2005).

Likewise, NMDARs also interact with integrins. The interaction between integrins and the extracellular matrix or potentially adjacent cellular ligands increase the amplitude of NMDAR currents (Lin, Arai, Lynch, & Gall, 2003). These findings demonstrate that the function of Sxc and NMDARs are impacted by the cellular adhesion protein integrin and that perhaps, Sxc and NMDARs are

interacting with the same ligands within the extracellular matrix. A key experiment would then be to determine if Sxc and NMDARs co-localize or co-immunoprecipitate via their association with integrins. If so, it would suggest that glutamate signaling within the extrasynaptic compartment follows similar design principles to the synaptic cleft by utilizing transcellular protein bridges that facilitate high-fidelity intracellular communication between release mechanisms and receptors.

As introduced above, the extracellular matrix may permit the creation and maintenance of discrete signaling zones. Another critical factor in the formation of discrete signaling zones is the volume of the interstitial space, which is determined by the physical proximity of cellular membranes (often astrocyte and neuronal membranes). Notably, the volume of the interstitial space is regulated by neuronal activity, in part by influencing astrocyte swelling. In support, neuronal depolarization causes an elevation in extracellular K+ which triggers uptake into adjacent astrocytes through inwardly rectifying K+ channels and Na+/K+ ATPase. This process produces an osmotic gradient in astrocytes that drives water influx through aquaporins causing the astrocytes to swell and thus reduce extrasynaptic volume (Hubel & Ullah, 2016). Additionally, glutamate released by neurons drive uptake though astrocytic transporters e.g. GLT-1 which co-transports Na^{+}. This also triggers swelling in the astrocyte and consequently a reduction in the extrasynaptic volume (MacAulay, Hamann, & Zeuthen, 2004), which can produce an anisotropic diffusion pattern i.e. a biased directionality to the flow of glutamate in the extracellular space. Such cellular barriers reduce the

mobility of glutamate by producing “dead zones” in the extracellular space that impede the flow of signaling molecules and bias glutamate access to specific zones within the extracellular domain (Vargová & Syková, 2014). Given these findings, synaptic glutamate (or glutamate released into extrasynaptic microdomains) could be compartmentalized from synaptic glutamate via cellular swelling. These ideas will require further research, but they provide a clear demonstration that a more thorough understanding of the complexity within the tripartite may lead to highly novel approaches to understand glutamate signaling in health and disease.

The Significance of Sxc to Cognition

Our studies provided multiple examples of cognitive dysfunction following a loss of Sxc. We found that MSxc rats more readily engage with a reward-paired CS (CS+) during the PCA paradigm. Intriguingly, approach toward CS+ was maintained even if this behavior was punished by withholding reward delivery. This was the first hint that MSxc rats displayed cognitive deficits, which was illustrated by an inability to adapt their behavior in response to changing reward contingencies and is considered a principle executive function (Diamond, 2013). This type of executive dysfunction was also observed during the attentional set shifting paradigm, whereby MSxc rats took significantly more trials relative to WT rats to adapt a new behavioral strategy to obtain rewards in a maze. Likewise, in Kamin fear blocking, attentional gating of extraneous stimuli was disrupted in MSxc rats which made them more likely to freeze to stimuli that were otherwise weak predictors of danger. These maladaptive associative learning and inflexible

perseverative patterns of behavior seen in MSxc rats manifested as an increased vulnerability to relapse of cocaine seeking.

The role of Sxc in cognitive processing appears to depend on the complexity of cognition or behavior. As noted above, a loss of Sxc impacted cocaine reinstatement, reversal learning, extra-dimensional shift, goal-tracking, and Kamin blocking. In contrast, it did not alter the formation of simple Pavlovian or operant associations, novelty-induced locomotion, cocaine self-administration, extinction, or fear conditioning. In comparing behaviors impacted versus those that are unaltered, it appears that behaviors impacted by a loss of Sxc are generally deemed to be more complex and involve top-down processing. These data are similar to the pattern of behavior observed by disruption of evolutionarily-new components of the synaptic proteome (Nithianantharajah et al., 2013). Collectively, these findings lend support to the idea that evolutionarily novel biological mechanisms, including those expressed by astrocytes, may provide novel opportunities to understand and treat cognitive dysfunction.

Can a Phylogenetic Strategy Expedite the Development of Effective CNS Therapeutics?

As is the case for most forms of human disease, a key therapeutic breakthrough may not be realized until the molecular basis of the pathological process is understood to the point of being able to develop effective and safe treatments. For CNS disorders, this is exceedingly challenging. Uncovering the basis of cognition is hindered by the exceptional complexity of the brain, which makes developing therapies that avoid producing significant side effects challenging. For example, the prior sections provide a sense of the complexity

that has been revealed within a single tripartite; presumably, this is far from complete. Regardless, scaling up the complexity inherent in a single tripartite to account for the billions of cells and trillions of signaling microdomains provides a useful perspective of the challenge posed by the human brain through reductionist approaches. This has led some investigators to search for a different approach to understanding and treating cognitive dysfunction.

One effective strategy may be to leverage phylogenetics, the study of the evolutionary relationships among species at the systemic and molecular level (Z. Yang & Rannala, 2012). From this we may be able to discriminate foundational neural mechanisms from those that may be selectively involved in enabling phylogenetic gains in cognition. To implement this approach, genes that were recently acquired or currently evolving within the vertebrates can be manipulated to determine their biological significance (E. J. Vallender, 2008). For example, adaptations in the intracellular domain of the GluN2 subunit of NMDA receptors expanded the complexity of its downstream signaling partners, which may explain the expansion of cognitive ability in vertebrates versus invertebrates (T. J. Ryan, Emes, Grant, & Komiyama, 2008).

Similarly, the four paralogues of *Dlg*/membrane-associated guanylate kinase scaffolding protein in the synapse have evolved in vertebrates to have discrete roles in cognitive processing. For example, *Dlg1/4* share the greatest sequence homology with the ancestral invertebrate *Dlg* and when knocked out in mice can produce unviable mice or significant deficits in basic learning whereas *Dlg2/3* knockouts have a more unique sequence relative to ancestral *Dlg*

resulting in no deficits in basic learning but only cognitive deficits during complex tasks (S. G. Grant, 2016). This suggests that phylogenetics can be used to isolate biological components and categorize them based on their involvement in fundamental or adaptive brain functions. Identifying these adaptations will be critical if we are to understand the molecular origins of what separates our cognitive ability from other organisms, and may lead to rapid gains in our ability to treat cognitive dysfunction.

Closing Remarks

Substance abuse, depression, anxiety, cancer, type 2 diabetes, cardiovascular disease, obesity, and schizophrenia represent the most debilitating diseases in the modern world. To tackle this disease burden from a utilitarian perspective and do the greatest good for the greatest number of people possible, we need to approach treating disease by tackling the common underlying symptoms. The common symptom in the above disorders is cognitive dysfunction (Cannon et al., 2017; Harrison & Wefel, 2018; He et al., 2018; Jones et al., 1992; Lange et al., 2017; Millan et al., 2012; P. M. Moran et al., 2008; Rock et al., 2014; Y. Yang et al., 2018). Each of these disease states carry an inherent inability for the patient to appropriately adapt their behavior in response to their internal thoughts and emotions and external environmental circumstances which produces an overall reduction in their biological fitness. Solving this problem will take a lot of work, and this document represents a paradigm shift in how we can investigate novel therapeutic avenues. Using principles from evolutionary biology and expanding beyond the neuron and synapse, we can hopefully more easily

determine what the molecular basis of cognition in humans is and thus be more effective in treating dysfunctional states. While this work is far from complete, it sets the foundation for research into other mechanisms expressed by astrocytes that may also contribute to cognitive disorders. While Sxc serves as an intriguing therapeutic target a concession can be made that Sxc and more broadly astrocytes may not be *the* solution, but it serves as a well needed paradigm shift in how we view brain function and how to treat its most debilitating diseases.

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