Regulation of Mouse Hippocampal Synaptic Transmission by Glial xCT

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THESIS

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“Be bold and the mighty forces will come to your aid.”

-Johann Wolfgang von Goethe
This thesis is dedicated to my husband Mark Richard James Williams and my mother Kathleen Walters. It has been your unconditional love and acceptance that has made this work possible.
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<td>aCSF</td>
<td>Artificial Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>a.u.</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>APDC</td>
<td>(2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C°</td>
<td>Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CPG</td>
<td>Carboxyphenylglycine</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>CY5</td>
<td>Cyanine 5</td>
</tr>
<tr>
<td>Cys-Cys</td>
<td>cystine</td>
</tr>
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<td>CysSH</td>
<td>cysteine</td>
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<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>Gb</td>
<td>Genderblind</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
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<tr>
<td>HATs</td>
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KS test  Komolgrov-Smirnov test
LTD     Long Term Depression
LTP     Long Term Potentiation
M       Molar
MAGUK   Membrane-Associated Guanylate Kinase
mEPSC   Miniature Excitatory Postsynaptic Currents
min     Minutes
mIPSC   Miniature Excitatory Postsynaptic Currents
ms      Millisecond
mL      Milliliter
mV      Millivolts
N       Number
NAC     N-acetylcystine
nM      Nanomolar
nL      Nanoliter
NBQX    2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-
        dione
NMDAR   N-methyl-D-aspartate receptor
pA      Picoamps
PBS     Phosphate-buffered saline
PCP     Phencyclidine
pF      Picofarads
PFA     Paraformaldehyde
PKC     Protein Kinase C
PKA  Protein Kinase A
PSD  Postsynaptic density
ROS  Reactive Oxygen Species
SAP-102  Synapse Associated Protein
s  Seconds
SEM  Standard Error to the Mean
sEPSC  Spontaneous Excitatory Postsynaptic Currents
T840-P  T840-phosphorylated
TRITC  Tetramethylrhodamine
TTX  Tetrodotoxin
vs.  versus
xCT  System Xc⁻
μM  Micromolar
CHAPTER I: INTRODUCTION
1.1 Chapter Summary

This chapter will discuss the neurotransmitter glutamate and postsynaptic glutamate receptors. It will emphasize that there are two pools of glutamate in and around glutamatergic synapses: the vesicular and the nonvesicular. Over half of the nonvesicular pool of glutamate originates from cystine-glutamate antiporters or System X_c- and they will be described in detail. Their role at the synapse is central to this thesis. I will describe cysteine-glutamate’s structure, kinetics, cystine’s role intracellularly, their location in the brain, and how the transporter is regulated. I will then describe the xCT/- loss of function mouse model I used in this analysis and what has been found thus far phenotypically in mutant System X_c- mice. Finally, I will describe our lab’s previous work in Drosophila which demonstrates that the xCT transporter releases a constitutive extracellular pool of glutamate that normally causes a suppression of postsynaptic transmission. Later chapters will build upon the Drosophila work by demonstrating a similar suppression mechanism in the mouse hippocampus.

1.2 Glutamatergic Synapses

1.2.1 Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the brain. Glutamate is an incredibly important synaptic neurotransmitter and is formed from α-ketogluterate, an intermediate of the krebs cycle. Once it is produced it is packaged into vesicles and released into the synaptic cleft upon arrival of an action potential (Hodgkin and Huxley, 1945; Krebs et al., 1948; Heuser and Reese, 1973; Sudhof, 1989; Zorumski et al., 1996).
Up to 80% of synapses in the mammalian brain are glutamatergic. Changes in synaptic glutamate signaling are the basis of many forms of learning, memory, as well as some mental disorders. Not surprisingly, synaptic glutamate signaling is intensely studied, and increasingly well understood (Kau et al., 2008; Knackstedt et al., 2010; Moussawi and Kalivas, 2010; Lewenz et al., 2013).

1.2.2 Postsynaptic Glutamate Receptors

There are two types of glutamate receptors located at the postsynaptic membrane, namely, ionotropic and metabotropic. Metabotropic receptors are characterized by their use of G-proteins, second messengers, and cascades which can impact many cellular mechanisms in both the long- and short-term (Sugiyama et al., 1989; Masu et al., 1991; Bortolotto and Collingridge, 1992; Upreti et al., 2013).

Conversely, ionotropic receptors are channel forming receptors which open and close their channel in response to neurotransmitter binding. Channel opening allows certain ions to flow into or out of the postsynaptic cell. The type and the movement of ions through ionotropic receptors are based on the characteristics of the channel and the electrochemical gradient across the postsynaptic cell membrane (Mullins, 1961; Diamond and Huxley, 1968; Neher and Sakmann, 1976). These experiments in this thesis will focus on ionotropic glutamate receptors; however, it has been demonstrated and hypothesized that metabotropic glutamate receptors (mGluR) may also be affected by and can modulate cystine-glutamate transporters (Reissner et al., 2014; Scofield and Kalivas, 2014) (Baker et al., 2002; Baker et al., 2003).
There are three types of ionotropic glutamate receptors: 1) the N-methyl-D-aspartate (NMDAR) receptor, 2) the kainate receptor, and 3) the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) receptor (Yamazaki et al., 1992; Sun et al., 1994).

The AMPAR is primarily responsible for mediating fast synaptic transport in the brain because it opens and closes rapidly and once opened can quickly become desensitized. Its name comes from an artificial specific agonist AMPA and it primarily transports the cations Na⁺ and K⁺ through its channel pore. Each AMPAR has 4 glutamate binding pockets, one for each subunit. AMPARs are composed of 4 different subunits GluR1 (GRIA1), GluR2 (GRIA2), GluR3 (GRIA3) and GluR4 (GRIA4). Most receptors at mature synapses are heterotetrameric consisting of two GluR2 subunits and two GluR1, GluR3, or GluR4 (Wenthold et al., 1992; Bochet and Rossier, 1993; Sun et al., 1994).

This analysis will focus specifically on AMPA receptors; however, it is reasonable to suspect that the NMDA or kainate receptors could also be affected by cystine-glutamate transporters.

1.3 Two pools of glutamate at glutamatergic synapses: vesicular and nonvesicular

1.3.1 Vesicular glutamate (Figure 1)

The concentration of glutamate in the cytoplasm of neurons is 5-10 mM. It is thought to be 2-3 times that at axon terminals because axon terminals are rich in mitochondria and mitochondria are high in glutaminases which make glutamate (Ly and Verstreken, 2006).

At the glutamatergic synapse, the concentration of glutamate inside synaptic vesicles is about 100 mM (Ottersen, 1989; Shupliakov et al., 1992; Featherstone, 2010). Arrival of an action
potential at the presynaptic buton triggers synaptic vesicles to release neurotransmitter into the synaptic cleft (Clements et al., 1992; Featherstone, 2010). Following vesicular release glutamate concentrations within the cleft rise to 1-3 mM within less than 100μs and return to ambient concentrations (0.5-5 μM) within 0.5-5 ms. Glutamate receptors are rapidly activated in response to this sudden rise in agonist (Clements et al., 1992; Clements, 1996; Bergles et al., 1999; Dzubay and Jahr, 1999; Danbolt, 2001).

The Excitatory Amino Acid Transporters (EAATs) are primarily responsible for rapid reuptake of glutamate following vesicular release and are responsible for the timescale of the postsynaptic response. Astrocytes at synapses have a high density of EAATs specifically EAAT1 (GLT-1, SLC1A2) and EAAT2 (GLAST, SLC1A3) (Bristol and Rothstein, 1996; Bergles and Jahr, 1997; Bergles et al., 1999). Once glutamate is taken up by astrocytes it is turned into glutamine by glutamine synthase. Glutamine is then transported back into neurons by asc-1, the transporter also responsible for cysteine uptake in a variety of cells, and deaminated back into glutamate (Rothstein et al 1996, Kilberg 1981 & 1980). This cytoplasmic glutamate is then repackaged into vesicles via the vesicular glutamate transporters located on synaptic vesicles (Takamori, 2006).

1.3.2 Nonvesicular Glutamate (Figure 1)

Most extracellular glutamate in the brain, however, arises from non-synaptic mechanisms (Timmerman and Westerink, 1997; Jabaudon et al., 1999; Shinohara et al., 2000; Baker et al., 2002; Fillenz, 2005; Montana et al., 2006; Leichsenring et al., 2013). This non-synaptic glutamate appears to regulate a variety of phenomena in the brain, including cell differentiation, cell migration, and synaptic strength via a variety of mechanisms, including
direct action on synaptic glutamate receptors as well as modulation of metabotropic receptor activity (LoTurco et al., 1995; Nguyen et al., 2001; Baker et al., 2002; Demarque et al., 2002; Manent et al., 2005; Manent and Represa, 2007; Grosjean et al., 2008; Scofield and Kalivas, 2014).

Blood plasma glutamate concentration has been measured to be about 150 μM and glutamate concentration in human Cerebral Spinal Fluid (CSF) has been shown to be around 10 μM. However, many studies have suggested that there is a constitutive, nonvesicular, extracellular glutamate in the intact mammalian brain that is between 0.5 to 5 μM (Herra-Marschitz 1996, Miele 1996, Baker 2002, Kottegoda 2002, Kennedy 2002, Day 2002).

Nonsynaptic glutamate can be released from neurons and glia, via a variety of molecular mechanisms. For example glutamate can be released nonvesicularly via swelling activated anion channels, gap junction hemichannels, and purinergic receptors (Herra-Marschitz et al., 1996; Miele et al., 1996; Zorumski et al., 1996; Baker et al., 2002; Kennedy et al., 2002; Kottegoda et al., 2002; Featherstone and Shippy, 2008). However, in vivo microdialysis experiments in rats, mice, and Drsosophila suggest that over half of all nonsynaptic glutamate is attributable to cystine-glutamate antiporter activity (Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011).
Figure 1: How postsynaptic glutamate receptors access glutamate. A representative schematic of a synapse which depicts a presynaptic bouton facing a postsynaptic dendritic spine. The two are separated by the synaptic cleft. The postsynaptic dendritic spine has several ionotropic glutamate receptors. Neuroglia, probably astrocytes, surround the synaptic cleft. Glutamate receptors have access to glutamate via two mechanisms: 1) the extensively studied release via synaptic vesicles, which raises cleft glutamate concentrations to 1-3 mM. Glutamate receptors are rapidly activated in response to this high concentration of glutamate. Glutamate is quickly taken back up by the EAATs located on astrocytes which account for the 0.5-1.5 millisecond (ms) timescale of vesicular release dependent responses. 2) In contrast, xCT, suggested to be located on glia, constitutively releases a micromolar (0.5-5 μM) concentration of glutamate that is nonvesicular in origin.
1.3.3 Loss of cystine-glutamate antiporters and extracellular glutamate concentration (Figure 2)

Genetic ablation of the xCT transporter in *Drosophila* results in a decrease in ambient extracellular glutamate to less than half of controls (Augustin et al., 2007). A similar decrease (50-70%) in extrasynaptic glutamate is seen with loss of the xCT transporter in rodents, either genetically removed or via xCT inhibitor induced blockade, in the striatum, nucleus accumbens, and hippocampus (Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011) (Melendez 2005). Similarly, reverse microdialysis of N-acetylcystine (N-Ac), an xCT transporter pro-drug, *in vivo* into the rat brain significantly enhances extracellular glutamate levels (Baker et al., 2002; De Bundel et al., 2011). Extracellular glutamate in mammals is insensitive to tetrodotoxin, a blocker of presynaptic action potentials, and is calcium independent which demonstrates it is nonvesicular in origin (De Bundel et al., 2011; Featherstone, 2011; Massie et al., 2011).

These results suggest that cystine-glutamate antiporters or System $X_c^-$ contributes to over half of the ambient extracellular glutamate found in the brain and this is conserved across organisms (Jabaudon et al., 1999; Shinohara et al., 2000; Baker et al., 2002).
Figure 2: Loss of System X_c causes a decrease in extracellular glutamate to less than half of controls. a) Larval hemolymph relative concentration of glutamate from microdialysis measurements in Drosophila. Precise excision controls contain the background of the genderblind (gb) mutants. Genderblind is the Drosophila homolog of System X_c. KGO7905 refers to the P-element insertion responsible for genderblind gene disruption. (b) Glutamate concentration in the striatum of sut/sut mice, a naturally arising xCT transporter loss of function mutant, compared to SnJ background strain. There was significant decrease in extracellular glutamate in sut/sut mice compared to controls (*). (c) Glutamate concentration in xCT-/ mice, the mutant strain used in this analysis, compared to controls. Measurements taken via microdialysis in vivo in the intact mouse hippocampus. There was a significant decrease in extracellular glutamate in xCT-/ mice compared to controls (*). Images modified from (Augustin et al., 2007; De Bundel et al., 2011; McCullagh and Featherstone, 2014)
1.4 Cystine-glutamate antiporters or System X_c also known as the xCT transporter

1.4.1 What are cystine-glutamate antiporters?

Bannai and Kitamura in 1980 first identified a cystine transport system whose activity was inhibited by glutamate in mammalian cultured cells and vice versa (Bannai and Kitamura, 1980). The xCT transporter or System X_c exchanges intracellular glutamate for extracellular L-cystine at a 1:1 molar ratio (Sato et al., 1999; Sato et al., 2000; Sato et al., 2002). Cystine and glutamate are transported in their anionic forms and the transporter exhibits stereo specificity to L-type substrates. Homocysteate, α-aminoadipate, and α-aminopimelate are also substrates for xCT (Bannai, 1986).

X-ray diffraction of System X_c (Figure 3) has revealed the transporter is a heterodimer composed of a “heavy chain” 4F2hc (90 kDa) subunit which is responsible for function and surface expression, and a “light chain” xCT (40kDa) subunit, which is responsible for excitatory amino acid specificity (Bassi et al., 2001; Gasol et al., 2004; Palacin et al., 2005). The xCT subunit is specific to System X_c and is a 502 amino acid protein. It is highly hydrophobic and contains 12 transmembrane domains. The 4F2hc subunit is a cell surface type II glycoprotein with one transmembrane domain and is known to form heterodimers with at least 5 other excitatory amino acid transporters. The xCT transporter is a member of the Heteromeric Amino Acid Transporter (HATs) all of which are composed of a heavy (SLC3 gene family) and a light (SLC7 gene family) subunit (Mastroberardino et al., 1998; Verrey, 2003; Verrey et al., 2004).

The two subunits are held together by a single conserved cystine residue (amino acid residue 158), found in both human and mouse xCT, which is consistent with other 4F2hc transporters. This cystine residue is located in the loop between the third and fourth
transmembrane domains (Sato et al., 1999; Bassi et al., 2001). The 4F2hc subunit is required for the xCT subunit to reach the plasma membrane and in the absence of the 4F2hc subunit xCT remains intracellularly (Bassi et al., 2001).
**Figure 3: System X\textsubscript{c} - and a model for all Heteromeric Amino Acid Transporters.**

Drawing of System X\textsubscript{c} based upon the crystal structure of the protein. 4F2hc is the heavy chain subunit (90 kDa), which induces surface expression. The 4F2hc is common to many HATs. The 12 transmembrane barrels are a part of the light chain xCT (40kDa) subunit which is responsible for excitatory amino acid specificity. The two subunits are held together by a single conserved cystine residue (amino acid residue 158), found in both human and mouse xCT, which is consistent with other 4F2hc transporters. Figure adapted from (Gasol et al., 2004).
1.4.2 xCT Transporter Kinetics

The inward flow of extracellular cystine is largely due to the outward flow of extracellular glutamate and movement would be close to zero without intracellular glutamate (Bannai, 1986; Bassi et al., 2001). Transporter activity is independent of membrane potential (Bannai, 1986). Direction of exchange is determined by the substrate concentration gradient with micromolar levels of L-cystine extracellularly and millimolar levels of intracellular L-glutamate (Danbolt 2001, Sagara 1993 a). Cystine is rare in the cytosol because it is rapidly reduced to L-cysteine, glutathione, and a mixed disulfide of glutathione and L-cysteine (Bannai and Kitamura, 1980; Sato et al., 1999). L-glutamate remains relatively unchanged once released extracellularly.

cDNA injected in to Xenopus oocytes with the human SLC7a11 and the 4F2hc genes resolved the K\text{m}, or the substrate concentration when the reaction rate is half of V\text{max}, of the transporter for glutamate to be 92±14 \text{µM} and for cystine 43±2 \text{µM}. V\text{max}, the max rate of the transporter, for glutamate is 164±7 pmol/15 min per oocyte and for cystine 50±1 pmol/10 min per oocyte. The Hill coefficient, a measure of a change in affinity for substrate in response to substrate binding, for L-glutamate is 1.12±0.09 and for L-cystine is 1.04±0.04. A Hill coefficient of 1 indicates substrate binding does not enhance or decrease the transporter’s affinity for more substrate which is consistent with the 1:1 molar ratio exchange observed for xCT (Bassi et al., 2001). Similar kinetic values were obtained from human fibroblasts. (Bannai and Kitamura, 1980)
1.4.3 The origin of cystine and its use intracellularly

While this analysis will largely focus on glutamate released extracellularly and its effect on postsynaptic glutamate receptor abundance. It is important to understand System Xc\(^-\) as a whole, since the release of glutamate extracellularly is dependent upon availability of cystine.

Cystine is a disulfide formed between two cysteine molecules. L- cystine is taken up by System Xc\(^-\) (Figure 4) and is quickly reduced to cysteine due to the highly reducing intracellular environment (Bannai, 1986). The transport of cysteine, the reduced form of cystine, is primarily mediated by the asc-1 transporter and lesser by Lat-2 which is a bidirectional transport system (Verrey et al., 2004). Cysteine is continuously being driven from the cell by asc-1 or Lat-2 because it autoxidizes to cystine extracellularly. This cycle is being driven by not just metabolism and cellular need but by the redox state extracellularly (Bannai and Ishii, 1982).

Extracellular cystine is generated from: oxidation of cysteine released from cells, from release by glutathione (GSH), imported from plasma or synthesized from the transulferation of methionine (Aoyama 2011, McBean 2011). Cystine is present in blood levels at 5-10 fold higher levels than cysteine (Droge et al 1991). Hepatocytes, unlike other cells, actively synthesize cysteine from methionine and serine via the cystathione pathway and use it for GSH synthesis (Bannai, 1986). Dietary cystine uptake in the intestines and reabsorption in the kidneys are important for maintaining required levels of the amino acid in the body (Burdo 2006). Cystine in human cerebral spinal fluid (CSF) is in the range of 0.25-1.3 μM and 4 μM in rats (Sato et al., 2002).

The intracellular cysteine is a rate-limiting step in glutathione synthesis. GSH concentration in human CSF is between 5-6 μM (Sato et al., 2002). Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine (kato 1993).
Glutathione synthesis occurs via a two part mechanism intracellularly:

_Gamma glutamyl transferase_

(Reaction 1) L-glutamate + L-cysteine + ATP $\leftrightarrow$ y-L-glutamyl-L-cysteine + ADP + P\textsubscript{i}

_Glutathione synthase_

(Reaction 2) y-L-glutamyl-L-cysteine +glycine $\leftrightarrow$ GSH + ADP + P\textsubscript{i}

(Bannai, 1986)

Reaction 1 is inhibited by GSH (Bannai, 1986). Glutathione is both an antioxidant enzymatically, GSH peroxidases can convert harmful hydrogen peroxides to H\textsubscript{2}O and O\textsubscript{2}, or nonenzymatically as a direct electron donor (Burdo 2006).

Loss of the xCT transporter in mice results in diminished GSH levels in cultured cells and blood plasma (Sato et al., 2005). However, invivo in the intact mouse brain measurements do not show diminished GSH levels and the underlying cause of this apparent contradiction is not clear (De Bundel et al., 2011).
Figure 4: Schematic of System $X_c^-$ transport and the cystine-cysteine cycle. System $X_c^-$ takes up cystine (CyS-CyS) intracellularly and puts out glutamate (Glu) at a 1:1 molar ratio. Once cystine is taken up intracellularly it is rapidly converted to cysteine (CySH) due to the high reducing environment intracellularly. Cysteine is used by the cell for glutathione and protein synthesis. Cysteine can be released extracellularly or taken into the cell by the Neutral Amino Acid Transport Systems usually asc-1. When cysteine is released extracellularly it is oxidized to cystine due to the oxidizing environment extracellularly. This specific depiction is of cultured cells; however, the same mechanism applies to *in vivo* model systems. Figure modified from Bannai 1989.
1.5 System \( X_c \) genetics and mice loss-of-function mutations

1.5.1 The xCT gene \( SLC7a11 \)

The human xCT subunit gene or \( SLC7a11 \) is located on chromosome 4q28-31 between D4S2429 and D4S2659 (Figure 5) (Sato et al., 2000; Bassi et al., 2001). Human xCT shows a 93% similarity and 89% identity to the mouse xCT transporter (Sato et al., 1999; Bassi et al., 2001). The mouse xCT transporter is located on chromosome 3 also mapping to the \( SLC7a11 \) gene (Chintala 2005). The \( SLC7a11 \) gene consists of 12 exons and 78,256 bps (Sato 2011).

The gene for the heavy chain subunit 4F2hc, which induces surface expression, is \( SLC3A2 \) (solute carrier family 3 member 2). The human gene has been mapped to chromosome 11q13 (Teixeira et al., 1987).

1.5.2 Genetic loss- of- function xCT/- mouse model

The xCT/- model used in this analysis was genetically engineered by Sato in 2005. The ATG start codon, between two \( Ncol \) sites, is removed and replaced with an eGFP cassette. There are no xCT mRNA transcripts or protein found in this strain (Figure 5) (Sato et al., 2005; McCullagh and Featherstone, 2014).

There are two other xCT transporter loss- of- function mouse strains: \( sut/sut \) and xCT mu/mu. The \( sut/sut \) mutants were first identified as mutants due to their naturally occurring pigment mutation, the subtle gray (\( sut \)) mutation, which exhibits a yellowish coat phenotype. It was later discovered that the coat color is actually due to loss of cystine which System \( X_c^- \)
transports into the cell. This model demonstrates that genetic mutations resulting in xCT transporter loss of function can naturally arise. The mutation is a 481,280 bp deletion whereby exon 12 is replaced with a downstream start codon. No mRNA transcripts were found in *sut/sut* mice. No functional protein is found in this strain (Chintala et al., 2005; McCullagh and Featherstone, 2014).

Our lab has performed breeding crosses of xCT-/- and sut/sut mice which results in no functional protein. However, in these double mutants there was no observable decrease in extracellular glutamate in the striatum as seen in either xCT-/- or sut/sut. It is not yet exactly clear why this decrease was not observed (McCullagh and Featherstone, 2014). The third loss-of-function model was mutated by N-ethyl-N-nitrosurea mutagenesis. This resulted in a loss of the last 3 exons of the gene due to an in-frame STOP codon in exon 10 (Nabeyama et al., 2010).
Figure 5: xCT loss-of-function models in mice. Representative image of the SLC7A11 gene in control mice. Depictions of the xCT−/−, sut/sut, and xCTμ/μ gene mutations. This analysis was a comparison of the xCT−/− mice to controls with normal gene function. The background controls of this strain are C57BL/6J. Figure adapted from (Conrad and Sato, 2012),
1.6 xCT Transporter Location

1.6.1 xCT transporter expression in the body

Experiments localizing the xCT transporter to specific areas of the body show the most robust expression of SLC7a11 xCT mRNA to the CNS, including: the cerebellum, cerebral cortex, medulla, putamen, cerebral ventricles, meninges, area postrema, the subfornical organ, habenular nucleus, hypothalamus, and the spinal cord (Sato et al., 2002). Western immunoblotting experiments have localized the transporter protein to the cortex, paraventricular organs, hippocampus, striatum, to a lesser extent in the cerebellum, and all major brain regions (Shih et al., 2006). Outside of the brain further comparative RT-PCR analysis detected xCT mRNA in the mouse intestines and human intestinal epithelium. A faint signal has localized xCT to mouse kidneys but not human kidney cell lines, however, the HEK293 cell lines from the human pancreatic cell line show SLC7a11 expression (Sato et al., 2000; Bassi et al., 2001; Sato et al., 2002).

1.6.2 Cell types with xCT transporter expression

The xCT transporter protein has been demonstrated in mice to be largely located on astrocytes, microglia, Müller cells (glial cells of the retina), ependymal cells, glioma cells, and immature cortical neurons. Mature neurons exhibit little or no xCT activity and presumably accumulate L-cystine through alternative systems (Zerangue and Kavanaugh, 1996; McBean and Flynn, 2001; Pow, 2001; Chen and Swanson, 2003; Verrey, 2003). In Drosophila xCT is
located on a previously undefined subset of glia with no overlap on neurons (Augustin et al., 2007).

Antibodies, however, for the xCT transporter have been difficult to engineer for use in brain slices because the xCT transporter is a highly lipophilic protein with 12 transmembrane domains (Figure 3) (Mastroberardino et al., 1998; Bassi et al., 2001; Verrey, 2003; Verrey et al., 2004). Pow 2001 cleverly used an antibody against α-aminoapidate a specific inhibitor of the xCT transporter, not normally found in rodent brains, to detect its intracellular accumulation. Accumulation of the substrate was largely found in glia, not neurons and was specifically localized to Bergman glia, radial glia, and astrocytes. Staining was not found in oligodendrocytes (Pow, 2001).

Consistent with the idea that System X_{c^-} is located on neuroglia, over half of the unusually high glutamate transport in human glioma cell lines is attributable to the xCT transporter, and this high transport can be inhibited by the xCT inhibitor carboxyphenylglycine (CPG) (Ye et al., 1999). Similarly, under tissue culture conditions cystine uptake appears to be associated with glial cells but not neurons (Saraga 1993, Pow2001).
1.7 xCT transporter regulation

1.7.1 xCT transport regulation

The xCT transporter is induced by oxygen, electrophilic agents, and depleted cystine (See Figure 3) (Bannai, 1984; Bannai et al., 1989). In human cultured fibroblasts transport of cystine into the cell is highly diminished in a low (3%) oxygen environment and could be restored to normal levels when then incubated under room air; this was dependent on xCT transporter protein up regulation (Bannai et al., 1989).

The addition of electrophilic agents to cultured cells, such as diethyl malate, at high concentrations (1mM) enhanced the transporters activity by an increase in the $V_{\text{max}}$ and this is dependent on RNA and protein synthesis. Several electrophilic agents were tested with similar results. It is known that these electrophilic agents deplete glutathione and GSH is protective for the cell against electrophilic attack (Bannai, 1984).

In macrophages it has been demonstrated that the lipoprotein (oxi-LDL) is a potent inducer of the transporter. Oxi-LDL is known to be engulfed by macrophages under severe oxidative stress and eventually can lead to atherosclerotic lesions. When macrophages engulf oxi-LDL there is an increase in glutathione production from increased cystine transport through the xCT transporter on the macrophage plasma membrane. This increased glutathione contributes to the cells antioxidant protection from oxidative stress (Sato et al., 1995).

Lipopolysaccharide or bacterial endotoxin is also an activator of the xCT transporter and is found in the outer membrane of gram negative bacteria. In the presence of bacterial
endotoxin, macrophages and microglia release excitotoxic glutamate via the xCT transporter as a way to attack the invading bacteria (Kigerl et al., 2012).

1.7.2 xCT transporter inhibitors and activators

System Xc\(^{-}\) is inhibited by L-homocysteate, quisqualate, carboxyphenylglycine (CPG), sulfazalazine and extracellular glutamate (Figure 6). It is difficult to make inhibitors for the transporter because its antagonist pharmacology can affect glutamate receptors (Patel 2004). The xCT transporter only diverges from the GluR2 subunit of the AMPA receptor by having a lipophilic binding domain (Bridges 2012). The xCT transporter is negatively regulated by group II metabotropic glutamate receptors, specifically mGluR2/3, via a cAMP-dependent protein kinase. There is a potential cAMP-dependent phosphorylation site at Thr-45 on the transporter (Kim 2001).

In cultured rat astrocytes APDC ((2R, 4R)-4-Aminopyrrolidine-2,4-dicarboxylate) a metabotropic glutamate receptor (mGluR2/3) agonist (1000 \(\mu\)M) greatly decreased transporter activity. However, at lower concentrations (1 and 10 \(\mu\)M) APDC significantly increased the uptake of cystine. LY341495 (1\(\mu\)M) an mGlu2/3 antagonist showed a significant reduction in transporter activity in cultured astrocytes. H89 a PKA inhibitor, also showed a bell-shaped response curve where at 10 nM the transporter is stimulated and at 1 \(\mu\)M the transporter is inhibited. The PKA activator forskolin inhibited xCT transporter activity. Chelerythrine chloride, which eliminates PKC activity, increased transporter activity. Finally, inhibition of calcium/calmodulin-dependent kinase II with KN93 increased transporter activity. This highlights a possible mechanism to modulate activity in a bidirectional way that suggests a
dependence on concentration, where at certain concentrations the transporter can be activated and at others inhibited via the same mechanism (Tang and Kalivas, 2003).

Inhibition of system $X_c^-$ by high glutamate concentrations extracellularly causes glutathione depletion, oxidative stress and cell death in culture (Chen 2000; Maher & Davis 1996). $N$-acetylcysteine (N-Ac) a precursor of cysteine and extracellular cystine itself is known to activate System $X_c^-$ (DeBundel 2011).
Figure 6: Protein structure of the System Xc\textsuperscript{-} and activators and inhibitors of the transporter. Blue and purple colored activators/inhibitors are targeted to the cystine uptake and glutathione pathway. Pink are considered inhibitors of overall transport. Red are inhibitors/activators of the mGluR2/3 glutamate receptors. Lipopolysaccharide (green) activates the transporter which is thought to provide excitotoxic protection against bacteria. This figure was modified from its original version from (Verrey et al., 2004).
1.8 Loss of System X\textsubscript{c}  

1.8.1 *In vitro* System X\textsubscript{c} studies

Loss of the xCT transporter in melanocytes, fibroblasts, meningeal cells and astrocytes cultured from xCT mutant mice display very little or no cystine uptake (Chintala et al., 2005; Sato et al., 2005; Shih et al., 2006). Glutathione is also diminished in melanocytes cultured from xCT mutant mice (Chintala et al., 2005). For cultured cells from xCT mutant mice to survive and proliferate, the addition of β-mercaptoethanol to the culture media is required. Loss of the transporter causes loss of a constant source of cystine into the cell. This cystine, as previously discussed, is required for the production of glutathione, a crucial cellular antioxidant. The β-mercaptoethanol provides thiols or a reducing environment or more cysteine extracellularly. This cysteine, the reduced form of cystine, can then be taken up into the cell by an alternative transport system, either asc-1 or Lat-1 (Chintala et al., 2005; Shih et al., 2006). The death of these cells is mediated by stress mediated pathways like CHOP and eIF2, it occurs apoptotically, and involves the activation of c Jun N-terminal kinase caspases-3/-9 (Qiao 2008). The addition of the antioxidant Vitamin E to culture media is sufficient to allow xCT mutant cultured fibroblasts to survive but not proliferate (Sato et al., 2005). Similarly, hematopoietic cells derived from xCT mutant mice could not be stimulated to become macrophages or dendritic cells, the antigen-presenting cells of the immune system, without the presence of β-mercaptoethanol (Nabeyama et al., 2010).
1.8.2 Phenotypes of loss of xCT in Mice and Rats

In contrast to in vitro studies, in vivo xCT is apparently dispensable for the viability and survival of xCT-/− mice. Mutant xCT mice are healthy in appearance and fertile (Sato et al., 2005). However, while this has not been fully assessed, xCT-/− survivability immediately following birth appears to be diminished and mothers seem to have a prevalence towards eating their newborn pups. In our colony we have employed a system of using control mothers to surrogate xCT mutant pups which appears to increase survivability.

Behaviorally, xCT-/− mutant mice exhibit altered spatial working memory (De Bundel et al., 2011), even though it remains unclear whether this is definitively attributable to loss of xCT (McCullagh and Featherstone, 2014). xCT mutant mice also exhibit impaired fear and passive avoidance memory (Li et al., 2012).

As expected with loss of a constitutive source of cystine transport into the cells xCT mutants exhibit higher plasma cystine levels and lower plasma GSH concentrations. Unexpectedly, xCT mutants do no exhibit lower GSH content or an increase in oxidative stress markers in the brain (De Bundel et al., 2011). As previously discussed, xCT mutant mice in vivo also exhibit decreased ambient extracellular glutamate to less than half of controls in the brain areas of the striatum and the hippocampus (De Bundel et al., 2011; Massie et al., 2011; McCullagh and Featherstone, 2014).

Other phenotypes in xCT mutant mice include impaired macrophage survival and increased tumor proliferation which indicates that the xCT transporter has a protective effect on inflammation (Nabeyama et al., 2010). Mutant xCT mice were found to be highly protected against substantia nigral neurodegeneration when induced by 6-hydroxydopamine and had decreased susceptibility to limbic seizures (De Bundel et al., 2011; Massie et al., 2011). As
previously mentioned, the sut mice exhibit a mutant subtle gray pigmentation. Further analysis has shown that reduced cystine uptake into melanocytes, due to loss of the xCT, reduces pheomelanin pigment production causing the subtle grey coat color (Chintala 2005).

Electrophysiologically, Li et al (2012) took field recordings at hippocampal CA3-CA1 synapses of xCT mutant mice and reported reduced LTP, suggesting altered synaptic physiology (McBean, 2012).

In this thesis I evaluated xCT mutant mice electrophysiologically via whole-cell patch-clamp electrophysiology which has never been done in any of the xCT mutant mice lines or with the use of inhibitors. I will show that xCT mutant mice exhibit an increase in postsynaptic receptors and increased synaptic transmission in the hippocampus.

1.8.3 Loss of the xCT transporter in Drosophila

_Drosophila’s_ xCT homolog was identified by our lab and was named _genderblind_ due to the mutants interesting behavioral phenotype. Genderblind mutant males court and attempt to mate with other males. This genderblind behavior can be turned on with inducible RNAi suggesting that the phenotype is not the result of development but of nervous system control (Grosjean et al., 2008).

As previously discussed, similar to xCT mutant mice genderblind _Drosophila_ exhibit a significant decrease in ambient glutamate to less than half of controls indicating that the transporter is a major regulator of extracellular glutamate levels (Piyankarage et al., 2010; De Bundel et al., 2011). They also exhibit a significant increase in postsynaptic glutamate receptors (Augustin et al., 2007; Chen et al., 2009). Further investigation has demonstrated a mechanism by which glutamate released by xCT causes constitutive desensitization and down regulation of
synaptic glutamate receptors (Grosjean et al., 2008; Piyankarage et al., 2008; Chen et al., 2009). As expected for a potent modulator of synapse strength, genetic manipulation of xCT was subsequently shown to cause profound changes in behaviors previously thought to be 'hard-wired' (Piyankarage et al., 2008).

This analysis will build upon our laboratory’s previous work and methods used in *Drosophila*. The question of this thesis addressed whether xCT’s suppression of postsynaptic receptors is conserved in mice. Alternatively, is there an increase in post synaptic receptors in xCT-/- mice?
CHAPTER II: HYPOTHESIS
2.1 Chapter Summary

This chapter will outline the hypothesis that ambient extracellular glutamate released by glial xCT causes a suppression of the number of receptors. I will describe how this suppression process works when the xCT transporter is functioning properly. I will describe Drosophila experiments showing that the down regulation of postsynaptic receptors is dependent upon glutamate binding to receptors and is dependent upon receptor desensitization. I will then describe what happens when there is loss of xCT transporter function. This causes a significant decrease in ambient extracellular glutamate and an increase in postsynaptic receptor abundance. This has been demonstrated in Drosophila and in the present study will show a comparable effect in xCT-/− mice. Finally, I will describe why the CA3-CA1 hippocampal synapse in mice is a good model system for exploring this gap in knowledge.

2.2 Ambient extracellular glutamate’s effect on postsynaptic glutamate receptor abundance

2.2.1 Two major lines of evidence suggest that glial xCT regulates glutamatergic synapse strength.

The first line of evidence comes from drug studies in rats. Accumulating evidence suggests that reinstatement of drug seeking might involve increased glutamatergic transmission from the prefrontal cortex to the core of the nucleus accumbens (Kalivas, 2009; Scofield and Kalivas, 2014). Repeated exposure to addictive drugs leads to down regulation of xCT in the nucleus accumbens, which in turn is thought to reduce extracellular glutamate, lower metabotropic glutamate receptor (mGluRs) activity on the cortical presynaptic terminals to the nucleus accumbens core, reduce glutamate release, and trigger drug-seeking (Kalivas, 2009; Madayag et al., 2010; Scofield and Kalivas, 2014). Consistent with this model, activation of cystine-glutamate exchange with N-acetylcysteine or treatment with mGluR 2/3 agonists reduces
Secondly, our lab’s investigations have confirmed a *Drosophila* xCT homolog ('genderblind') as the major source of extracellular glutamate in flies and demonstrated a mechanism by which glutamate released by xCT causes constitutive desensitization and down regulation of synaptic glutamate receptors (Grosjean et al., 2008; Piyankarage et al., 2008; Chen et al., 2009). As expected for a potent modulator of synapse strength, genetic manipulation of xCT was subsequently shown to cause profound changes in behaviors previously thought to be 'hard-wired' (Piyankarage et al., 2008).

### 2.2.2 Two ways glutamate receptors have access to glutamate (Figure 1)

To reiterate, glutamate receptors have access to glutamate via the two pools previously discussed and they are vesicular and nonvesicular release. Vesicular release has been extensively studied and is increasingly well understood. It has a rapid (0.5-5) millisecond timescale and releases a relatively high concentration of glutamate into the synaptic cleft. Vesicular release at the glutamatergic synapse raises the concentration of cleft glutamate to 1-3 mM and this rapidly activates glutamate receptors (Clements et al., 1992; Clements, 1996; Bergles et al., 1999; Dzubay and Jahr, 1999). Glutamate is quickly taken up by the EAATs, located in high density on astrocytes (Clements et al., 1992; Clements, 1996; Bergles and Jahr, 1997; Bergles et al., 1999) (Bristol and Rothstein, 1996; Sutherland et al., 1996; Amara and Fontana, 2002).

Comparatively, System X\textsubscript{c}⁻, hypothesized to be located on glia, releases a constitutive, nonvesicular glutamate which contributes more than half of the extracellular glutamate ranging in concentration from 0.5-5 μM. This glutamate has been demonstrated to be nonvesicular in
origin. It has not yet been demonstrated via whole-cell patch-clamp electrophysiology in mice what this micromolar glutamate does to the synapse and that will be addressed in this thesis (Timmerman and Westerink, 1997; Jabaudon et al., 1999; Shinohara et al., 2000; Baker et al., 2002; De Bundel et al., 2011).

2.2.3 Ambient extracellular glutamate desensitizes receptors

Glutamate at ambient concentrations, 0.5-5 μM, can trigger activation of NMDA receptors (Figure 6) (Patneau & Mayer 1990, Williams 1994, Wyllie 1996, Nahum-Levy 2001 Herra-Marshitz 1996, Miele 1996, Baker 2002, Kottegoda 2002, Kennedy 2002, Day 2002). However, activation may not occur at all without membrane depolarization, due to the nature of the NMDA receptor, and/or in response to a constitutive low concentration of glutamate present at the synapse.

Compared to activation, steady-state desensitization of ionotropic glutamate receptors occurs at much lower concentrations (Figure 7) (Featherstone & Shippy 2008). If you evaluate suggested desensitization curves for glutamate receptors when glutamate is at ambient concentrations three-fourths of all NMDA, and one-half of all AMPA and kainate receptors will be desensitized (Colquhoun et al., 1992; Zorumski et al., 1996; Jones et al., 1997; Paternain et al., 1998).
Figure 7: Activation of receptors at ambient extracellular glutamate concentrations. Constitutive glutamate receptor activation versus various ambient extracellular glutamate concentrations (μM). The line indicates the measured ambient extracellular glutamate concentrations. Over 40% of all NMDA receptors and over 10% of all mGlu receptors will be constitutively activated in response to ambient extracellular glutamate concentrations. Image kindly provided by Featherstone and Shippy (Featherstone and Shippy, 2008).
**Figure 8: Desensitization of receptors at ambient extracellular glutamate concentrations.**

Constitutive glutamate receptor desensitization or percentage of non-desensitized receptors versus various ambient extracellular glutamate concentrations (μM). The line indicates the measured ambient extracellular glutamate concentrations. Over 75% of all NMDA receptors and nearly half of all AMPAR and kainate receptors will be constitutively desensitized in response to ambient extracellular glutamate concentrations. Image kindly provided by Featherstone and Shippy (Featherstone and Shippy, 2008).
2.2.4 Receptor desensitization triggers loss of postsynaptic receptors

Our lab has demonstrated in *Drosophila* that when receptors are desensitized they are functionally removed from the synapse (Augustin et al., 2007). Consistent with our results Zorumski et al. has confirmed, in cultured rat hippocampal neurons, that bath applied ambient glutamate (0.5 μM) depresses the amplitude of excitatory postsynaptic currents (EPSCs) and decreases the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Zorumski 2006). Application of extracellular glutamate for 24 hours to semi-intact wild-type larvae typically reduces glutamate receptors immunoreactivity to 1/3 of normal (Figure 7). Genetic manipulation of glutamate metabolizing enzymes can alter the number of postsynaptic glutamate receptors at the *Drosophila* neuromuscular junction and non-vesicular glutamate regulated receptor clustering (Featherstone et al., 2000; Featherstone et al., 2002; Piyankarage et al., 2008). The consequence of this mechanism of desensitization and removal is suppression of synaptic transmission in the fly (Zoromski 1996, Tardin 2003, Priel 2006, Augustin 2007).

*Thus the most likely effect of ambient glutamate at a concentration between 0.5- 5 μM is a decrease in synaptic transmission to less than one-half what it might otherwise be without steady-state desensitization.*

2.3 Requirements for receptor suppression by ambient extracellular glutamate

2.3.1 Glutamate receptor suppression requires glutamate binding

Our lab has also demonstrated in *Drosophila* that glutamate binding is required for desensitization and suppression of receptors. At *Drosophila* neuromuscular junctions there are two types of glutamate receptors: GluRIIA and GluRIIB (Figure 9). There is a mutant strain of
Drosophila where all GluRIIA receptors contain an E783A mutation which disrupts glutamate binding to the receptor. This disruption prevents both activation and desensitization of receptors. Application of extracellular glutamate for 24 hrs to semi-intact control larvae typically reduces glutamate receptor immunoreactivity to 1/3 of normal. This was again seen in the GluRIIB receptors of GluRIIA E783A mutants because GluRIIB receptors are not affected by this mutation, thus Glutamate can bind to the GluRIIB receptors. In contrast, no change in GluRIIA immunoreactivity was observed compared to NMJs which were not bathed in extracellular glutamate. We therefore conclude that glutamate binding is required for this suppression mechanism.

2.3.2 The receptor suppression by glial xCT requires receptor desensitization

Our lab has demonstrated in Drosophila that when larval NMJs are co-cultured with ambient extracellular glutamate and Concavalin A (a potent inhibitor of glutamate receptor desensitization) receptor suppression is also blocked (Figure 10) (Augustin et al., 2007). Receptor desensitization is therefore required for receptor suppression.
Figure 9: Glutamate binding is required for receptor suppression. (a) Bar graph with synaptic GluRIIA synaptic immunoreactivity when larval control Drosophila NMJs are bathed without glutamate (0 mM) and in 2 mM Glutamate. At 2 mM there is a significant decrease in immunoreactivity. The E783A mutation disrupts glutamate binding to the GluRIIA ionotropic receptor. When E783A NMJs are bathed in 2 mM glutamate receptor suppression is blocked. (c) Representative images of GluRIIA immunoreactivity and NMJs of the data described in (a). (b) E783A mutants have normal GluRIIB receptors. Immunostaining of GluRIIB receptors after bath application of 2 mM glutamate results in synaptic receptor suppression. (d) Representative NMJ immunoreactivity images of the data depicted in (b). Figure kindly provided by (Chen et al., 2009)
Figure 10: Receptor suppression requires desensitization. A) Schematic of the open, closed and desensitized states of ionotropic receptors. For the AMPAR in mammals and the GluRIIA/GluRIIB in *Drosophila* there are 4 glutamate binding pockets. Concavalin A blocks desensitization. B) Bar graph with synaptic GluRIIA synaptic immunoreactivity when larval control *Drosophila* NMJs are bathed without glutamate (0 mM) and in 2 mM glutamate. At 2 mM there is a significant decrease in immunoreactivity. The Concavalin A disrupts glutamate receptor desensitization. When Concavalin A is added to NMJs in 0 mM compared to 2 mM glutamate receptor suppression is blocked. (c) Concavalin A also blocks receptor suppression for GluRIIB receptors. Figure kindly provided by (Augustin et al., 2007)
2.3.3 Summary: ambient extracellular glutamate triggers postsynaptic receptor suppression

(Figure 11)

Nonvesicular glutamate release by the xCT transporter causes desensitization of receptors and this is dependent upon glutamate binding to receptors. Receptor desensitization triggers removal or decreased clustering at the postsynaptic membrane. It has not yet been demonstrated whether receptor down regulation actually prevents receptors from clustering at the synapse or if receptors are actually removed. Further experiments are needed to make this determination.

2.4 Loss of xCT transporter function and postsynaptic glutamate receptor abundance

As previously discussed loss of the xCT transporter causes on average a 60% decrease in ambient extracellular glutamate in both mice and flies (Figure 12). In Drosophila genderblind mutants exhibit a decrease in extracellular glutamate to half of controls (Augustin et al., 2007). In xCT-/- mice ambient glutamate is similarly decreased in the hippocampus and the striatum (De Bundel et al., 2011; McCullagh and Featherstone, 2014). Reverse dialysis of CPG (an inhibitor of xCT) into the nucleus accumbens also results in a significant decrease in extracellular glutamate (Baker et al., 2002; Baker et al., 2003).

Decreased glutamate concentration causes a decrease in extracellular glutamate, and this lowers the probability that receptors will become desensitized (Featherstone, 2010). Decreased receptor desensitization causes an increase in the number of receptors at the synapse. Genderblind or xCT-/- mutant Drosophila show an increase in functionally available receptors as measured electrophysiologically by significantly increased sEPSCs amplitude compared to controls. Genderblind mutants also showed a 200-300% increase in ionotropic glutamate receptor immunoreactivity (Augustin et al., 2007; Grosjean et al., 2008; Chen et al., 2009).
Figure 11: The xCT transporter causes desensitization and removal of receptors. (a) Illustration of the effect of xCT transporter released ambient extracellular glutamate (0.5-5 μM), on postsynaptic glutamate receptors. At this concentration a large percentage of receptors will be desensitized. (b) Once receptors are desensitized they are removed from the synapse.
Figure 12: Model, loss of the xCT-/- transporter causes decreased extracellular glutamate, desensitization, and removal of postsynaptic receptors. Loss of the xCT transporter causes a decrease in ambient extracellular glutamate to over half of controls. Less extracellular glutamate availability, due to loss of xCT, causes less desensitization of receptors. Receptor desensitization, which is required for receptor removal, is reduced. Therefore, loss of the xCT transporter causes more receptors at the synapse.
2.5 The gap in knowledge

Unfortunately, the *Drosophila* work has not been confirmed in mammals, and the rat work relies on pharmacology and inferences about cellular physiology that have not been directly observed. It has not been previously demonstrated in mice whether ambient extracellular glutamate affects postsynaptic glutamate receptor abundance in mice.

2.6 The hippocampal CA3-CA1 synapse as a model system

I addressed this gap in knowledge by using electrophysiology and immunohistochemistry to measure glutamatergic transmission and glutamate receptor abundance in hippocampal CA3-CA1 synapses from xCT mutant mice. There are several reasons why I choose this particular synapse. First, CA3-CA1 synapse physiology is well described and behaviorally relevant. Second, xCT mRNA expression is particularly strong in the mouse hippocampus, and xCT activity has been observed specifically in astrocytes surrounding CA1 neurons (Pow, 2001). Third, extracellular glutamate concentrations are reduced by 60% in the hippocampus of xCT mutant mice, there is no detectable cell death in the hippocampus of xCT mutant mice, and the glutamate measured in the hippocampus is not calcium dependent, consistent with the idea that extracellular glutamate in the hippocampus is primarily regulated by xCT (De Bundel et al., 2011). Fourth, xCT-/- mutant mice show altered spatial working memory (De Bundel et al., 2011), which depends on hippocampal function, even though it remains unclear whether this is definitively attributable to loss of xCT (McCullagh and Featherstone, 2014). xCT mutant mice
also exhibit impaired fear and passive avoidance memory both which are hippocampus-dependent learning paradigms (Li et al., 2012).

Finally, Li et al (2012) reported reduced LTP in hippocampal CA3-CA1 synapses from xCT mutant mice, suggesting altered synaptic physiology. This phenotype was not replicated by acute exposure to the xCT blocker 4-carboxyphenylglycine (CPG) (Li et al., 2012), but we wouldn't expect it to be if xCT glutamate modulated transmission via the mechanism shown in Drosophila, which takes hours to have an effect (Augustin et al., 2007).
3.1 Chapter Summary

This chapter will describe the Animal Care and Use in this analysis. I will discuss the electrophysiology slice preparation and the electrophysiology set-up. The voltage-clamp experiment and how mEPSCs, mIPSCs, sEPSCs, and EPSCs were obtained. It will describe how the immunohistochemical slices were prepared. It will go into detail about low flow push-pull perfusion and the electrophoretic glutamate concentration analysis.

3.2 Animal Care and Use

All procedures were reviewed and approved by the University of Illinois at Chicago Animal Care and Use committee. xCT knockout mice were generously provided by Hideyo Sato, Yamagata University, Japan (Sato et al., 2005). The xCT knockout mutation was generated in the C57BL/6J background and outcrossed with C57BL/6J more than 10 times. The xCT transporters expression is completely eliminated in these xCT-/- mice (Sato et al., 2005; McCullagh and Featherstone, 2014). Homozygous C57BL/6J mice were used as controls. GluR1 phospho-T840 'penta' mutant mice (Lee et al., 2007) were kindly provided by Rick Huganir’s lab at John’s Hopkins University.

3.3 Electrophysiology slice preparation

To obtain brain slices, postnatal day 14-22 mice were anesthetized with isoflurane. Isoflurane was taken up and dispensed via a transfer pipette into a 50 mL conical tube with a kim wipe at the bottom. The concocal tube and mice were placed in a Tupperware. The mice were anesthetized via inhalation of the isoflurane. The conical tube prevents the isoflurane from causing skin irritation. The tails of the mice were pinched to ensure appropriate analgesia and anesthesia. The mice were immediately decapitated with scissors.
A midline cut with a scalpel was made down the scalp to remove the skin (Figure 13a). Scissors were inserted into the middle of the cerebellum through the skull and a shallow cut was made down the midline of the skull towards the forebrain. Both sides of the skull were removed with tweezers allowing brain access. A scalpel was inserted down the midline of the brain to separate the two hemispheres. Both hemispheres were immediately removed and immersed in an ice-cold sucrose cutting solution (87mM NaCl, 2.5 mM KCl, 7 mM MgCl$_2$, 75mM Sucrose, 25mM glucose, 1.25 mM NaH$_2$PO$_4$, 26mM NaHCO$_3$, and 0.5 mM CaCl$_2$). Ice-cold sucrose-based cutting and storage solution is not required and is not always utilized for slice preparation, however, I found it was helpful for creating healthy slices and was employed in this analysis (Bischofberger et al., 2006).

Within four minutes the hippocampus was isolated and submerged in the ice-cold cutting solution to prevent cell death. With the medial part of the brain facing downwards the frontal lobe and any residual cerebellum were removed (Figure 13b). The hippocampus was isolated by a method described as the “Magic cut” by Bischofberger (2006) the $\beta$ angle was approximately 10$^\circ$ to obtain CA1 patchable cells. The $\beta$ angle refers to a cut that takes off 10% of the dorsal most part of the brain slicing from dorsa lateral toward rostral medial at a 10$^\circ$ angle. A 0$^\circ$ angle will result in CA3 patchable cells (Bischofberger et al., 2006).
Figure 13: Hippocampal brain removal and slice preparation. (a) (Left) Anatomy compass. (Right) Depiction of the mouse brain surgery. The blue dotted line depicts the midline incision, performed while the brain is in the skull, which separates the two brain hemispheres. The yellow line is a cut made to remove the cerebellum. Care should be taken to only take off the cerebellum so as not to damage the hippocampus. (b) (Left) Anatomy compass. (Right) The medial surface of one hemisphere is placed downwards. A cut described as the “Magic cut” is performed taking off the dorsal or top 10% of the brain. A straight downward cut will result in CA3 patchable cells. In this analysis a cut with a 10° angle was performed to obtain CA1 patchable cells. This cut dorsal most edge is placed downwards on the vibratome. A similar surgery and was performed to isolate the hippocampus for the immunohistochemistry experiments.
A dedicated vibrating microtome was used to prepare 300 μm thick hippocampal slices while submerged in the ice-cold sucrose-based solution. Slices were mounted to the vibratome bath with super glue that was regularly replaced to avoid movement during slicing.

Slices were then immersed in freshly prepared artificial cerebral spinal fluid (aCSF) at an osmolarity of 300-305 mOsm (124mM NaCl, 26mM NaHCO₃, 3mM KCl, 1.25 NaH₂PO₄, 1mM MgSO₄, and 10mM Glucose) at 35°C for 30-45 minutes, after which they were allowed to reach room-temperature (18-22°C). The osmolarity of all solutions was checked by the VAPOR vapor pressure osmometer from Wescor. Thanks to Dr. Simon Alford for allowing the use of this equipment.

Regular osmolarity checks are not required if the recipe is followed. However, on numerous occasions the osmolarity was slightly out of the 300-305 mOsm range and had to be adjusted. It was suggested during my training with other labs that an important factor for obtaining whole-cell patch clamp configuration is the relationship between the internal and external solution. The external solution osmolarity is suggested to be 10-15% higher than the internal solution. Here, the internal patch solution was at approximately 270 mOSM and the external solution’s osmolarity was from 300-305 mOSM. 95% O₂/ 5% CO₂ was bubbled through all solutions.

### 3.4 Electrophysiology Set-Up (Figure 14)

A parastaltic pump manufactured by masterflex was utilized to allow continuous perfusion into and out of the bath. For this specific pump adequate perfusion required that there was one inflow tube and cartridge to two outflow tubes and cartridges to ensure aCSF does not
get backed up and overflow the bath. Air-tight, drip chambers, created by a 10 mL syringe and epoxy, were employed at both the inflow and outflow to create even flow of aCSF into the bath and to prevent electrical noise that may result from a continuous salt solution flowing through tubing. Alligator clips were also added to the tops of the drip chamber and attached to any possible sources of noise and grounded to the rig and amplifier. It is imperative that air does not enter the inflow as this will add bubbles to the bath which will disrupt the ability to obtain and maintain a gigaohm seal required for adequate whole-cell access. Vacuum grease and dental wax were essential for this purpose.

Ground electrodes for both the head stage and the stimulator were silver chlorided wire or a silver chloride pellet soldered to a wire and attached to the back of the headstage or the stimulator.

Slices were held in place by a harp which was obtained from world precision instruments. During use of the harp loss of the strings for holding the tissue in place were fixed using pantyhose and super glue for restringing.

All glassware was cleaned with distilled water and was used for the sole purpose of electrophysiology solutions. Glassware was never cleaned with detergents to avoid membrane disruption and to maintain healthy cells.
Figure 14: Photo of the electrophysiology rig modified from a *Drosophila* rig to a mouse slice electrophysiology rig.
3.5 Electrophysiology

All electrophysiology recordings were obtained via whole-cell patch-clamp of CA1 pyramidal cells, using the blind patch technique which is commonly used in CA1 hippocampal recordings due to the density of cell bodies and a variable inability to visualize the cell. As the electrode was immersed in the bath positive pressure was added to avoid debris from blocking the electrode tip. Flow of intracellular solution from the electrode tip cleans off the surface of the cell and aids in gigohm seal formation. Recordings were performed in freshly prepared aCSF bubbled with 95% O₂/5% CO₂. Patch pipettes (5-8 mΩ) were filled with a 270 mOsm, 7.3 pH, internal solution containing: 120mM K-glucronate, 10mM KOH, 9mM KCl, 3.48mM MgCl₂, 10mM HEPES, 4mM NaCl, and 10mM HEPES. Patch solution was prepared beforehand, aliquoted into 1mL centrifuge tubes, and stored in a 4⁰ freezer until needed.

The blind patch technique involves advancing the patch electrode through the CA1 cell line, very slowly, until a drop in resistance is observed. This resistance drop was visualized in the lab bench feature in P-clamp, which during seal formation was initially set to apply a 1 mV voltage-step for 1 millisecond (ms). This was followed by a rapid release in positive pressure from the syringe to obtain a gigaohm seal.

As the resistance increases toward gigaohm magnitude the voltage step is increased to 10 mV. In tandem, the voltage was then slowly dialed down to resting membrane potential or -70mV which further aids in gigaohm seal formation. A slight suction of negative pressure was applied to obtain whole-cell access. If negative pressure application did not result in whole-cell access the “Zap” feature on the amplifier was used. The “Zap” injects a large hyperpolarizing voltage pulse (about 1.5 V) for about 0.5 ms. This pulse initiates dielectric breakdown of the membrane patch and ruptures the membrane allowing for whole-cell access.
Both negative and positive pressure were added to the tip of the electrode via a 10 mL syringe attached to a stop cock as a matter of preference. A smaller syringe was not used as sealing and whole-cell configuration was more difficult to obtain. Many experimenters prefer to apply air pressure with their mouth; however, I prefer the syringe technique as it provides specific pressure control.
Figure 15: Whole-cell patch clamp configuration. (a) Confocal image of the hippocampus Image shows the cell bodies of CA1, CA3 and the dentate gyrus. (b) Magnified CA1 confocal image from the area outlined by the box in (a). Representative depiction of an electrode and its placement for making recordings of mEPSCs, mIPSCs, and sEPSCs.
Good whole-cell access was confirmed by a drop in resistance, gigaohm seal formation, visualization of capacitive transients, leak current at or near zero and no lower then -400pA, and detection of mEPSCs when the miniature detection protocol was started. The Cm, leak, and time to patch were documented for all recordings at the time of the recordings.

Recording electrodes were made of silver (Ag) wires and surrounded by a boroscillate patch pipette filled with intracellular solution. The Ag wire was frequently dipped in bleach and washed off to coat the surface in Cl-. This allows for current to be transformed smoothly from the Ag wire coated with AgCl- to Ag on the wire and Cl- hydrated into solution.

3.5 Voltage Clamp

All recordings were performed in voltage-clamp mode (see Figure 16). In this type of experiment the voltage was controlled by the experimenter. This analysis of CA1 pyramidal neurons in the mouse produces currents ranging from about 5 pA for mEPSCs to events as large as 400 pA for stimulated EPSCs. When the voltage is clamped the capacitive current (or charging up of the cell membrane) is eliminated except for a brief time when you step to a new voltage. Except for this brief capacitive current the current is proportional to cell membrane conductance or the number of open channels. Current flow is determined by the transmembrane voltage so the voltage-clamp determines the opening and closing of channels. This is the opposite of current-clamp mode where a current is applied and the change in voltage is measured (Axon Instruments, 1993). The digital amplifier used here was an Axon Multiclamp Commander 700B. The acquisition software was oscilloscope was P-Clamp clampex with a digidata series digitizer for the computer.
Figure 16: Voltage Clamp Experiment. (Top) Depiction of a voltage step from membrane voltage (V) to V1. (Bottom) When the voltage is clamped the capacitive current (or charging up of the cell membrane) is eliminated except for a brief time when you step to a new voltage. Except for this brief capacitive current the current is proportional to cell membrane conductance or the number of open channels. Current flow is determined by the transmembrane voltage so the voltage-clamp determines the opening and closing of channels.
3.6 mEPSCs, mIPSCs, sEPSCs, and EPSCs

All mEPSCs were recorded from cells voltage-clamped at -70 mV, in bath solution containing 10 μM bicuculline (to block GABA A receptors) and 1 μM tetrodotoxin (TTX; to block presynaptic action potentials). Bath application of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX) was used as a control to confirm that mEPSCs were attributable to AMPA receptor activity. All drugs were mixed into 40 mL of bubbled aCSF and bath applied immediately following whole-cell access. After one minute of bath application recordings were started.

Miniature inhibitory postsynaptic currents (mIPSCs) were recorded from cells voltage-clamped at -100 mV, because the reversal potential of the GABA Cl- channel is at -70mV or resting potential, in bath solution containing 1μM TTX and 5 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; a competitive AMPAR antagonist). Bicuculline was bath applied as a control to ensure that mIPSCs were attributable to GABA-A receptor activity.

Spontaneous excitatory postsynaptic currents (sEPSCs) and excitatory postsynaptic currents (EPSCs) were recorded from cells voltage-clamped at -70 mV without bath application of TTX or other drugs. EPSCs were generated by delivering a stimulator-driven voltage pulse to CA3 axons via a patch electrode filled with extracellular aCSF.
Figure 17: Whole-cell patch clamp configuration and stimulation electrode placement. (a) Confocal image of the hippocampus, image shows the cell bodies of CA1, CA3 and the dentate gyrus. (b) Magnified CA1 confocal image from the area outlined by the box in (a). Representative depiction of a recording electrode, a stimulating electrode and their placement for recording EPSCs.
All recording and analysis was performed using PClamp 10 software (Molecular Devices). The mEPSCs and mIPSCs were detected and measured using the Event Analysis tools in PClamp 10 Clamp fit software. The mEPSC and mIPSC amplitude and frequency measurements are averages from all events that occurred during 100-120 seconds of continuous recording. The same event analysis program was used for all traces mEPSCs, mIPSCs, and sEPSCs. EPSC amplitudes were measured by hand. Each replicate (N=1) represents data from one slice in one animal (thus, N always represents number of animals).

3.7 Immunocytochemical analysis and confocal microscopy

For immunohistochemical detection and quantification of synaptic proteins, mouse coronal brain sections were fixed in 4% PFA (Paraformaldehyde, pH 7.4) for 18-24 hours. Sections were cryoprotected overnight in phosphate-buffered saline (PBS) containing 30% sucrose and 0.02% NaN₃, then frozen at -20⁰C. For staining and imaging, 40 μm hippocampal slices were cut from the frozen sections on a sliding microtome, then incubated 1 hour free floating in a blocking solution of PBS (pH 7.4) containing 0.025% Triton and 5% Bovine Serum Albumin (BSA). After blocking, slices were incubated 18-24 hours in blocking solution containing primary antibodies. Primary antibodies used for this study include: Mouse anti- SAP-102 (1:500, UC Davis/ NIH NEUROMAB #75-058), Mouse monoclonal anti-synapsin (1:100, synaptic systems #106001), Rabbit anti- Glutamate Receptor 1, AMPA subtype phospho T840 (1:1000, abcam #ab12108), Rabbit Anti-Glutamate Receptor 1, AMPA subtype (1:500, abcam #ab31232).
After incubation in primary antibodies, slices underwent four 10-minute washes in PBS plus 5% BSA at room temperature. Slices were then incubated for 2 hours in PBS solution containing 5% BSA and appropriate fluorescently-conjugated secondary antibodies (1:400, Jackson ImmunoResearch, TRITC- and CY5-conjugated anti-mouse or anti-rabbit). Secondary fluorophores were switched for half of all preparations in each set of experiments, to control for possible differences in baseline fluorescence or secondary immunoreactivity. After incubation with secondary antibodies, slices were washed 4 times in PBS at room temperature and placed onto slides.

Images of Hippocampal Shaffer collateral-CA1 synaptic regions were generated using laser-scanning fluorescence microscopy at 540x magnification. The size of synaptic puncta was measured by manually delineating the edges of puncta using ImageJ software and calculating the enclosed area based on pixel dimensions. Only puncta where the GluR1 immunoreactivity (either GluR1 or T840) was colocalized with SAP-102 were used for measurements. Synaptic density was measured by counting the number of GluR1+SAP-102 puncta per μm². Each replicate (N=1) represents averaged data from 3 different slices from a single mouse (thus, N always represents number of animals).

3.8 Microdialysis

3.8.1 Low Flow Push-Pull Probe

The push-pull probe construction procedure was described previously (Piyankarage et al., 2008; Piyankarage et al., 2010; Pritchett and Shippy, 2014). Briefly, the sampling probes consist of concentric fused-silica capillaries that permit direct infusion of physiological saline to the
desired sample region via an outer infusion capillary (360/75 µm o.d./i.d) and simultaneous sample collection via an inner withdrawal capillary (150/75 µm o.d./i.d). These sampling arrangements were used in non-continuous mode for tissue sampling. The probe was mounted on a three axis high resolution micromanipulator for control and placement while sampling (World Precision Instrument, Sarasota, FL). 500 nL of extracellular fluid was collected via low flow push pull perfusion after the probe tip was placed 20µm inside of the CA1 region of a mice hippocampal slice.

3.8.2 Electrophoretic Assay

The conditions for the detection of glutamate in small volume samples were previously optimized in Dr. Scott Shippy’s laboratory (Figure 18). Electrophoretic analysis was performed on a laboratory-built CE system with a commercial high voltage power supply (Spellman, NY) and a ZETALIF detector (Picometrics, Paris, France) modified in-house with a LED 365 nm (Prizmatix, Modiin-IIite, Israel). All separations were achieved in 360/50 µm o.d./i.d. fused-silica capillary (Polymicro Technologies) with a total length of 50 cm (35 cm effective) at 27 kV applied potential. The CE capillary, buffer vials and electrode assembly were isolated with a plexiglasss box to separate the components and protect the operator. Before use, the capillary was conditioned with 1.0 M NaOH, deionized water and separation buffer at intervals of 45 to 60 seconds each. Samples were injected gravimetrically at 19 cm for 15 s allowing ~7.6 nL to be injected in to the capillary. Consecutive injections were performed from the same vial.
3.8.3 Data Analysis

Raw data were exported from a custom LabVIEW data acquisition program to Microsoft Excel in order to plot electrophoretic data. Peak heights were measured from electropherograms by subtracting the baseline, which was considered to be the average signal 1 s before the first peak of the electropherogram, from the peak maximum. The noise was calculated as the standard deviation of the same baseline section of the electropherogram. The signal-to-noise ratio (S/N) was calculated from the peak height divided by the standard deviation of the baseline. Concentrations were derived from calibration curves generated with standards.
Figure 18: Photo of the Low Flow Push-Pull Probe microdialysis set-up. Picture of the perfusion bath with a hippocampal slice being perfused. A harp holds the tissue in place. The probe, as seen here, is inserted into CA3-CA1 synapses at a 20 μm depth.
3.9 Statistical Analysis

Student's t-test was used for comparisons between two groups of normally distributed data. For analysis of data with unequal variances (as determined by a post-hoc F-test) a Mann-Whitney U test was used. A Kolmogrov-Smirnov test was used to compare distributions. For comparison of input-output curves (e.g. stimulation intensity vs. EPSC amplitude) an analysis of variance (ANOVA) was performed. Results were considered statistically significant when the calculated p-value ≤ 0.05.
CHAPTER IV: xCT-/- MICE EXHIBIT AN INCREASE IN POSTSYNAPTIC RECEPTORS
4.1 Increased amplitudes of mEPSCs in xCT-/- mice compared to controls

We hypothesized that loss of xCT would lead to accumulation of postsynaptic glutamate receptors. Arguably the most precise and quantitative method for detecting changes in postsynaptic glutamate receptors is measurement of miniature excitatory postsynaptic current (mEPSC) amplitudes. I therefore recorded mEPSCs from whole-cell patch-clamped (-70mV) CA1 pyramidal neurons in hippocampal slices (Fig. 19a). To ensure that the mEPSCs were due to glutamate receptor activity, the GABA-A receptor antagonist bicuculline was added to the bath. mEPSCs were also completely eliminated in the presence of 5 μM NBQX, demonstrating that they were due to AMPA receptor activity (Fig 19b).

Consistent with the idea that xCT -/- mutant synapses contain more glutamate receptors, mEPSCs recorded from xCT -/- mice were on average approximately ~37% larger than those recorded from C57BL/6J controls (control = 6.281 ± 0.3131 pA, N=8; xCT -/- = 9.892 ± 0.9401 pA, N=8). Interestingly, much of the increase in xCT -/- mEPSC amplitudes was attributable to an abundance of larger mEPSCs rather than a uniform shift in mEPSC amplitude (Fig 19d, e). This change in distribution was highly significant (Kolmogorov-Smirnov test, p< 0.0001). This is a logical consequence of receptor addition to synapses rather than genotype-specific differences in recording conditions or cell membrane characteristics.
Nevertheless, to rule out the possibility that mEPSC amplitude differences between genotypes might be due to other factors, we performed several additional measurements. First, we compared cell capacitance which is proportional to the size of the cell and efficacy of the voltage-clamp. It's conceivable that mEPSCs could appear larger if the cell was more completely voltage-clamped and would show up as an increase in cell capacitance. However, there was no difference in cell capacitance between xCT -/- mutants and controls (Fig. 19f). The quality of voltage-clamp, cell health, and/or receptor activity might also 'run down' over time. A systematic difference in the time that recordings were performed, after slice preparation, might also contribute to differences in mEPSC amplitude. However, there was no systematic difference in latency to onset of recording these measurements (Fig. 19g). It should be noted that all recordings included in this initial mEPSC analysis were taken within 1-3 hours after slice preparation in the event that experimental manipulation could alter glutamate concentrations extracellularly. Finally, effective voltage-clamp and recording of currents assumes minimal shunting of current due to factors such as: unhealthy, leaky cell membranes or poor patch electrode seals with the membrane. Such currents are unavoidable, but could contribute to differences in mEPSC amplitude if there was a systematic difference between genotypes. However, we observed no systematic difference in leak current between xCT -/- and control recordings (Fig 19h). We conclude, therefore, the larger mEPSCs in xCT -/- mutant mice, due to either an increase in postsynaptic glutamate receptors or an increase in glutamate receptor conductance.
Figure 19: mEPSC amplitudes are increased in xCT -/- mutant mice compared to controls. (a) Representative whole-cell voltage-clamp (-70 mV) recordings of control (black) and xCT -/- mutant (red) mEPSCs in CA1 of the hippocampus. Events were recorded in the presence of 1 μM TTX, to block presynaptic action potentials and 10 μM bicuculline to block GABA A receptors. Scale bars: horizontal 1000 ms, vertical 5 pA. (b) mEPSCs in the presence of 5 μM NBQX an AMPA receptor antagonist. (c) Box-and-whisker plots of mEPSCs amplitudes. The bottom and
top of the box are always the first and third quartiles, and the band inside the box is always the second quartile (the median). The + denotes the mean. mEPSC amplitudes recorded from xCT -/- mice which were on average approximately ~37% larger than those recorded from C57BL/6J controls (Mean (+ sign) ± SEM control = 6.281 ± 0.3131 pA, N=8; xCT -/- = 9.892 ± 0.9401 pA, N=8; t-test p=0.0027). (d) Cumulative probability distribution graph of raw mEPSC amplitudes. This change in distribution between genotypes was significant (Kolmogorov-Smirnov test, p<0.0001). (e) Histogram bar graph with bins at 0.5 pA. (f) mEPSC amplitude (pA) vs. cell capacitance (pF). (g) mEPSC amplitude (pA) vs. slice life in time (min). (h) mEPSC amplitude (pA) vs. leak current (pA) between xCT -/- and control recordings.

4.2 There is no increase in xCT-/- mIPSC amplitudes in xCT mice compared to controls

Does the change in xCT -/- mouse synapse strength apply to non-glutamatergic synapses? Our hypothesis provides no mechanism by which non-glutamatergic synapses would also accumulate receptors. To test this we recorded CA1 neuron mIPSCs, which are mediated by GABA receptors. This required the addition of the AMPAR blocker CNQX to the recording solution, and a change in holding potential to -100 mV, because the reversal potential of chloride is near -70 mV. Under these conditions, and the solutions used, mIPSCs are inward currents (Fig. 20a) that are blocked by the GABA A receptor antagonist bicuculline (Fig 20b). As expected, if the changes in xCT -/- mice are restricted to only glutamatergic synapses, we observed no change in mIPSC current amplitude (Fig. 20c-e; controls = 4.388 ± 0.8047 pA, n=6; xCT -/- = 5.379 ± 0.7760 pA, n=6; KS test p= 0.1484).
Figure 20: mIPSC amplitudes, or number of inhibitory receptors, are comparable in amplitude in xCT-/- mutant and control mice. (a) Representative whole-cell voltage-clamp (-100 mV) recordings from CA1 cells taken from control (black) and xCT-/- mutant (red) slices. Events were recorded in the presence of 1 μM TTX, to block presynaptic action potentials and 5 μM CNQX, a competitive AMPAR antagonist. (b) Bicuculline (10 μM) was bath applied as a control to ensure that mIPSCs were attributable to GABA A receptor activity. (c) We observed no change in mIPSC current amplitude (Mean (+ sign) ± SEM: controls = 4.388 ± 0.8047 pA, n=6; xCT-/- = 5.379 ± 0.7760 pA, n=6, Student t-test p= 0.3962). Box-and-Whisker plots. (d) Cumulative frequency distribution graph with no significant change in distribution across the two genotypes (KS test p= 0.1484). (e) Histogram bar graph with bins at 0.5 pA.
4.3 Glutamate receptors can be visualized in hippocampal slices.

Despite its precision and functional significance, electrophysiology cannot discern the difference between amount of postsynaptic glutamate receptor protein and changes in single receptor conductance. In *Drosophila*, loss of xCT leads to an increase in postsynaptic glutamate receptor proteins detectable by immunohistochemistry (Augustin et al., 2007).

GluR1 is a critical component of hippocampal CA3-CA1 synapses, and commercial antibodies that recognize it are easily obtained. However, hippocampal slices include multiple layers of overlapping cell processes and synapses, making reliable microscopic staining and identification of synaptic proteins notoriously difficult. Therefore, we developed methods for accurate quantitative visualization of postsynaptic glutamate receptors in slices. First, we ensured that fluorescent secondary antibodies alone did not contribute to staining that could be reasonably interpreted as receptor protein immunoreactivity. They did not (Fig 22). Next, we reasoned that relevant glutamate receptor immunoreactivity must lie adjacent to or colocalize with other synaptic markers, such as the presynaptic vesicle-associated protein synapsin, or the postsynaptic density (PSD) scaffolding protein SAP-102 (Fig. 21a). We chose SAP-102 because it is most abundant in the CA1 synapses of immature (P14-22) mice that were the focus of this study, it is replaced later in development by PSD-95 (Elias et al., 2006). As expected, GluR1 and
SAP-102 immunoreactivity was abundant in the region of slices containing CA1 dendrites, and most of it formed puncta within the size range expected for synaptic protein clusters (at least within the resolution of standard confocal microscopy; Fig. 22). However, substantial GluR1 and SAP-102 immunoreactivity was not overlapping (Fig. 22); consistent with the idea that some of the immunoreactivity was nonspecific (or at least nonsynaptic). Therefore, we restricted our analysis to the subset of GluR1 immunoreactivity that was overlapping with SAP-102 and adjacent to synapsin (Fig. 22).

As final confirmation that this GluR1 immunoreactivity truly represented postsynaptic GluR1 protein, rather than (for example) nonsynaptic GluR1 in dendritic spines, we used antibodies that specifically recognize T840-phosphorylated GluR1, which is thought to be found exclusively in postsynaptic densities (Lee et al., 2007). To ensure that the T840-P GluR1 staining was specific, we tested these antibodies in slices from T840A mutant mice, wherein GluR1 cannot be phosphorylated at this residue. In these T840A mutants, the T840-P antibody staining was reduced to background fluorescence levels (Fig. 22), suggesting that it is specific for T840-phosphorylated GluR1. Based on these experiments, we concluded that we could reliably quantify synaptic GluR1 immunoreactivity in hippocampal slices, as long as we were careful to restrict our analysis to the subset of GluR1 puncta that colocalize with SAP-102, or better yet the subset of T840-phosphorylated GluR1 puncta that colocalize with SAP-102.
Figure 21: Receptors can be visualized in CA3-CA1 synapses hippocampal slices. (a) Representative drawing of a presynaptic buton and postsynaptic dendritic spine. The diagram shows probable locations of the presynaptic protein synapsin, the postsynaptic marker SAP-102, the GluR1 subunit and T840-P GluR1 subunit of the AMPAR. (b) Top: Confocal image of a hippocampal slice. Representative overlay of the CA3-CA1 synapse. Scale bar: 50 μm. Bottom: GluR1(pink) and SAP-102 (green) immunostaining of synapses and (right) magnified. White puncta indicates overlap. Scale bar: 10 μm.
Figure 22: Staining of Individual Synaptic Puncta. Top: Staining of fluorescent secondary antibodies alone in control mice. Next: staining GluR1 (pink) and Synapsin (green). Next: staining of GluR1 (pink) and SAP-102 (green). Next: staining of T840-P GluR1 (pink) and synapsin (green). Bottom: staining of T840-P GluR1 (pink) and SAP-102 (green). Middle panel: T840A mutant slices stained with T840-P GluR1 (pink) and SAP-102 (green). Antibody staining
is mouse anti- SAP-102 (1:500), Mouse monoclonal anti-synapsin (1:100), Rabbit anti-Glutamate Receptor 1, AMPA subtype phospho T840 (1:1000), Rabbit Anti-Glutamate Receptor 1, AMPA subtype (1:500). Primary antibodies are conjugated to fluorescent secondary antibodies (1:400 TRITC- and CY5-conjugated anti-mouse or anti-rabbit). Scale bar: 1μm.

Figure 23 shows confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and GluR1. As expected, if loss of xCT leads to increased GluR accumulation, we measured a fairly large increase in the size of GluR1 immunoreactive puncta in xCT -/- mutant mice (Fig. 23a, b; xCT -/- = 0.4059 ± 0.043 um², n=8 mice; control = 0.5000 ± 0.055 um², n=8 mice; Student t-test p=0.1980). However, the average GluR1 immunoreactivity in this case was not statistically significant, probably because GluR1 staining represents both nonsynaptic and synaptic GluR1. However, the difference in the raw distributions of both genotypes was highly significant (Fig. 24, komolgrov- smirnov D= 0.2772, p<0.001). This is a logical consequence if some of the staining is a mixture of unchanged nonsynaptic GluR1 puncta and larger synaptic GluR1 puncta in xCT-/- mice.
Figure 23: Confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and GluR1 in xCT-/- mutant and control slices. (a) Top: GluR1 (pink) and SAP-102 (green) and overlap (white). Below: Just GluR1 (white) staining and representative depiction of how area measurements are taken (yellow dotted line). Puncta were only included in the analysis if they were overlapping the SAP-102. (b) GluR1 staining representing both nonsynaptic and synaptic GluR1 (Mean (+ sign) ± SEM xCT -/- = 0.4059 ± 0.043 μm², n=8 mice; control = 0.5000 ± 0.055 μm², n=8 mice; Student t-test p= 0.1980). The GluR1 immunoreactivity was not statistically significant. Box-and-whisker plot.
Figure 24: Cumulative frequency distribution of the GluR1 area for Control and xCT-/- immunoreactivity. The change in distribution between the two genotypes was highly significant (Kolmogrov-Smirnov D= 0.2772 p<0.001). Mutant xCT-/- have a larger fraction of larger area GluR1 puncta compared to controls.
**4.4 xCT-/− mice exhibit an increase in AMPAR receptor area at the synapse compared to controls.**

Figure 25 shows confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and either GluR1 or T840-phosphorylated GluR1. To restrict our analysis to synaptic GluRs, only GluR1 or T840-P GluR1 puncta that overlapped with SAP-102 were analyzed. Measurements using the T840-P GluR1 antibody show a statistically significant increase in the size of SAP-102-associated GluR1 immunoreactivity (Fig. 25c, d; control = 0.4207 ± 0.04291 μm², n=7 mice; xCT-/− = 0.6086 ± 0.06442 μm², n=6 mice; Student t-test p=0.0298). The magnitude of this postsynaptic T840-P GluR1 cluster size increase (~31%) is statistically indistinguishable from the increase in mEPSC size measured electrophysiologically (See Fig. 19). The distribution of the T840-P GluR1 puncta area was also significantly different between xCT-/− and control mice. Consistent with the student t-test xCT-/− have a larger fraction of events with larger puncta areas (Fig. 26, kolmogrov-smirnov, D=0.2556, p<0.001).
Figure 25: Confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and T840-phosphorylated GluR1 in xCT-/- mutant and control slices. (a) Top: Representative image of T840-P GluR1 (pink) and SAP-102 (green) staining. Bottom: T840-P GluR1 staining (white) and representative depiction of how area measurements are taken (yellow dotted line). Puncta were only included in the analysis if they were overlapping the SAP-102. (b) Area measurements show a statistically significant increase in the size of SAP-102-associated GluR1 immunoreactivity (Mean (+ sign) ± SEM control = 0.4207 ± 0.04291 μm², n=7 mice; xCT -/- = 0.6086 ± 0.06442 μm², n=6 mice; Student t-test p= 0.0298). Box-and-whisker plot and all scale bars: 1 μm.
Figure 26: Cumulative frequency distribution of the T840-P GluR1 area for Control and xCT-/- immunoreactivity. The change in distribution between the two genotypes was highly significant (komolgov-smirnov, D=0.2556, p<0.001). Mutant xCT-/- have a larger fraction of larger area T840-P GluR1 puncta compared to controls.
4.5 xCT/- mice do not exhibit an increase in AMPAR puncta intensity at the synapse compared to controls.

Puncta intensity was also measured and for the overall GluR1 staining there was not significant increase in the intensity between control (Fig. 27, Mean ± SEM: 319.8 ± 49.57 N=8) and xCT/- mice (306.5 ± 27.99 N=8, student t-test p= 0.8191). There was also no significant increase in the intensity of the T840-P GluR1puncta or receptors at the synapse between control (Fig. 28, 317.6 ± 35.56 N=7) and to xCT/- mice (295.2 ± 32.73 N=6, student t-test p= 0.6574). An increase in intensity would imply that there is an increase in the density of receptors rather than an increase in the size of the receptor field. No change in intensity suggests that the increase in receptors is due to additional receptors being added around the original receptor field as opposed to receptors being inserted into the original receptor field.

4.6 xCT/- mice exhibit an increase in SAP-102 MAGUK postsynaptic scaffolding area at the synapse compared to controls.

To address the possibility that the increase in GluR1 puncta size reflects an increase in postsynaptic density size we measured SAP-102 staining. The prototypical PSD- MAGUK
being PSD-95 and others in this family includes SAP-97 and PSD-93/Chapsyn-110. We chose SAP-102 because electrophysiological recordings in this study were made at immature synapses, postnatal day 14-22, and SAP-102 is the predominate MAGUK. SAP-102 is later replaced by PSD-95 at mature synapses (Elias et al., 2006).

Figure 31 shows confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and T840-phosphorylated GluR1. To restrict our analysis to protein at the synapse only SAP-102 puncta that overlapped with T840-P GluR1 were analyzed. Unexpectedly, the SAP-102 puncta area was also significantly increased in xCT-/- (Fig. 31, 0.6434 ± 0.1183 N=6) compared to control mice (0.4053 ± 0.03033 N=7, Mann-Whitney U p=0.0140). An F-test indicated that the xCT-/- and control SAP-102 puncta area had significantly different variances so a Mann-Whitney U was employed (F-test p = 0.0036). SAP-102 is in the postsynaptic density. It is a PDZ domain containing, membrane-associated guanylate kinase (PSD-MAGUK). Consistent with the area increase there was a highly significant change in the distributions of raw SAP-102 puncta area (Fig. 32, Kolmogrov-Smirnov D=0.4088, p<0.001).

SAP-102’s role in the postsynaptic density is to facilitate clustering of AMPA receptors. It is thus reasonable to expect that changing the number of receptors requires a change in the number of SAP-102 proteins (Elias et al., 2006). However, the increase in SAP-102 puncta size could also suggest that the effects of loss of the xCT transporter causes an increase in the average size of postsynaptic spines. This remains to be seen and will require ultrastructural analysis to determine whether the effect of loss of xCT on the postsynaptic dimensions, but we can clearly conclude that the number of receptors is increased.
Figure 27: Confocal images of CA1 dendritic regions in hippocampal slices, stained with antibodies against SAP-102 and T840-phosphorylated GluR1 in xCT-/- mutant and control slices. (a) Top: Representative image of T840-P GluR1 (pink) and SAP-102 (green) staining. Bottom: SAP-102 staining (white) and representative depiction of how area measurements are taken (yellow dotted line). Puncta were only included in the analysis if the T840-P GluR1 puncta overlapping the SAP-102 puncta. (d) Area measurements show a statistically significant increase in the size of SAP-102 immunoreactivity (Mean (+ sign) ± SEM control = 0.4053 ± 0.03033 N=7 mice; xCT-/- =0.6434 ± 0.1183 N=6, Mann-Whitney U p= 0.0140 0.6086 ± 0.06442 um², n=6 mice; Student t-test p= 0.0298). Box-and-whisker plot and all scale bars: 1 µm.
Figure 28: Cumulative frequency distribution of the SAP-102 area for Control and xCT-/- immunoreactivity. The change in distribution between the two genotypes was highly significant (Komolgrov-Smirnov, $D=0.4088, p<0.001$). Mutant xCT/- have a larger fraction of larger area SAP-102 puncta compared to controls.
CHAPTER V: FUNCTIONAL CONSEQUENCES
5.1 Chapter Summary

The electrophysiological and immunohistochemical data described in the previous chapter strongly suggest that CA3-CA1 postsynaptic glutamate receptor abundance is increased by approximately one-third in xCT -/- mutant mice. This is a significant change, on par with those thought to occur during processes such as learning and memory, and raises the possibility that xCT might be an important regulator of synaptic strength in vivo. However, a change in postsynaptic glutamate receptor abundance does not necessarily imply a physiologically-relevant change in synaptic function. The probability of presynaptic neurotransmitter release could drop to compensate for more receptors, for example, or the overall number of synapses between cells could be reduced. Furthermore, simulations of individual synaptic activity suggest that significant addition of postsynaptic glutamate receptors can have negligible effects on synaptic current amplitude, due to restrictions imposed by synaptic architecture and glutamate diffusion. This chapter will therefore examine whether xCT mutant mice show physiologically-relevant changes in CA3-CA1 synaptic function. Our results show that mutant xCT -/- exhibit increased sEPSC and EPSC amplitude and no change in synapse number.
5.2 xCT-/- mice have an increase in endogenous synaptic current compared to controls

First we measured spontaneous synaptic activity (sEPSCs) using the same procedures used to record mEPSCs, but leaving out the TTX to permit endogenous neuronal activity (Fig. 33). sEPSC amplitudes in xCT-/- mutant mice were significantly larger than those measured from controls (Fig 33 a, b; control = 10.36 ± 1.239 pA, n=9; xCT -/- = 16.54 ± 2.574 pA, n=7; Student t-test, p= 0.0354). Interestingly (and importantly), analysis of the sEPSC amplitude distributions showed that xCT -/- mutant mice sEPSCs were not larger on average just because a few larger sEPSCs occurred; the entire distribution appeared to be shifted toward larger events (Fig. 33 c,d; KS test D (0.4166), p< 0.0001, Fig. 33).
Figure 29: sEPSC amplitudes, as a measure of endogenous synaptic activity, are increased in xCT-/− mutants and controls in CA1 cells of the hippocampus. (a) Representative images of whole-cell patch-clamp (-70 mV) sEPSC traces in xCT-/− mutants compared to controls. Scale
bars: horizontal 1000 ms, vertical 5 pA. (b) Box-and-whisker plot of sEPSC amplitudes. In xCT -/- mutant mice sEPSCs were significantly larger than those measured from controls (Mean (+ sign) ± SEM control = 10.36 ± 1.239 pA, n=9; xCT -/- = 16.54 ± 2.574 pA, n=7; Student t-test, p= 0.0354). (c) Cumulative probability graph of sEPSC amplitude. Analysis of the sEPSC amplitude distributions showed that xCT -/- mutant mice sEPSCs were larger on average (KS test p< 0.0001). (d) Histogram bar graph of the sEPSC distribution.

5.3 xCT -/- mice exhibit an increase in the EPSC amplitude compared to controls

By the same argument, EPSCs generated by electrical stimulation of presynaptic inputs should be very large indeed, even with a relatively small uneven increase in postsynaptic GluR abundance. To test this, we used a patch electrode filled with extracellular bath solution to deliver electrical pulses to Schaffer collateral CA3 axons, while simultaneously recording EPSCs in postsynaptic CA1 cells. Consistent with the idea that the increase in GluRs observed in xCT -/- mutants would have disproportionate effects as more synapses are recruited, the median EPSC amplitude in xCT -/- mutants was approximately double that measured in controls (Fig. 34a,b; control = 115.6 ± 15.41 pA N=7, n=7; xCT -/- = 222.7 ± 47.18 pA, n=7; Mann-Whitney test p=0.0175). Also as predicted, the entire EPSC distribution was shifted (Fig. 34c,d; KS test D= 0.5015), as opposed to primarily having a larger tail.

To ensure that the changes in EPSC amplitude were not due to better efficacy of stimulation in xCT -/- mutants, we collected EPSC amplitudes at multiple stimulation intensities
(Fig. 34e). EPSC amplitude was consistently increased in xCT /- mutants independent of stimulation intensity (ANOVA p= 0.0017).
Figure 30: EPSC amplitudes are increased in xCT-/− mutants and controls in CA1 cells of the hippocampus. (a) Representative traces of EPSCs in xCT-/− mutant (red) and control (black) hippocampal slices. EPSCs are recorded by delivering electrical pulses to CA3 axons, while simultaneously recording EPSCs in postsynaptic CA1 cells. Scale bars: horizontal 100 milliseconds, vertical 50 pA. (b) Box- and- whisker plot of EPSC amplitude in xCT -/− mutants was approximately double that of controls (Mean (+ sign) ± SEM control = 115.6 ± 15.41 pA, N=7, n=7; xCT -/− = 222.7 ± 47.18 pA, n= 7; Mann-Whitney test p= 0.0175). (c) Cumulative distribution plot of EPSCs (KS test of distributions p<0.0001). (d) Bar graph histogram of the two genotypes binned at 20 pA. (e) EPSC amplitudes at multiple stimulation intensities. EPSC amplitude was consistently increased in xCT -/− mutants independent of stimulation intensity (ANOVA p= 0.0017).

5.4 xCT mice do not exhibit an change in the number of synapses compared to controls.
An increase in overall synapse number could also lead to larger sEPSCs and EPSCs. Therefore, we counted the density of CA1 synaptic GluR1 puncta in controls and xCT -/- mutants (Fig. 35a, b). We saw no significant change (control GluR1/SAP-102 puncta density = 0.01914 ± 0.002587/μm², N=8; xCT GluR1/SAP-102 puncta density = 0.01749 ± 0.001741/μm², N=8; p=0.6041; control T840-P GluR1/SAP-102 puncta density = 0.01431 ± 0.001479/μm², N=7; xCT T840-P GluR1/SAP-102 puncta density = 0.01386 ± 0.001260/μm², N=6; p=0.8245). If anything, xCT -/- mutants had slightly fewer synapses by this method.

Similarly, we observed no significant difference in mEPSC frequency between controls and xCT-/- mutants (Fig. 36, control = 0.2904 ± 0.08192 Hz, n=12; xCT = 0.3914 ± 0.2684 Hz, n=12; Student t-test p= 0.7224). We conclude, therefore, that the increased sEPSC and EPSC amplitudes measured in xCT -/- mutants are attributable to an increase in the number of postsynaptic GluRs.
Figure 31: No observed increase in number of synapses in xCT-/- mutant mice when counting number of synapses. (a) Representative images of xCT-/- mutant and control slices stained with SAP-102 (green) and either GluR1 or T840-P GluR1 (both pink). White puncta indicate overlap and were included in this analysis. Yellow arrows are representative overlapping puncta that would have been counted in the analysis. (b) Box-and-whisker plot of the density of CA1 synaptic GluR1 puncta in controls and xCT-/- mutants. We saw no significant change (Mean (+ sign) ± SEM in control GluR1/SAP-102 puncta density = 0.01914 ± 0.002587/μm², N=8; xCT GluR1/SAP-102 puncta density = 0.01749 ± 0.001741/μm², N=8; p=0.6041; control T840-P GluR1/SAP-102 puncta density = 0.01431 ± 0.001479/μm², N=7; xCT T840-P GluR1/SAP-102 puncta density = 0.01386 ± 0.001260/μm², N=6; p=0.8245).
Figure 32: No observed increase in number of synapses in xCT-/- mutant mice when measuring miniature frequency. (a) Representative mEPSC traces whole-cell voltage-clamped at -70mV in the presence of bicuculline and TTX. mIPSC traces whole-cell voltage-clamped at -100 mV in the presence of CNQX and TTX. Scale bars: horizontal 1 min and vertical 5 pA. (b) Box-and-whisker plot of mEPSC and mIPSC frequency. No significant difference in mEPSC frequency between controls and xCT-/- mutants (Mean (+sign) ± SEM control = 0.2904 ± 0.08192 Hz, n=12; xCT = 0.3914 ± 0.2684 Hz, n=12; Student t-test p= 0.7224). Red dot indicates a mEPSC frequency outlier.
CHAPTER VI: AMBIENT EXTRACELLULAR GLUTAMATE MODULATES POSTSYNAPTIC RECEPTOR TRAFFICKING

6.1 Chapter Summary
How does xCT suppress postsynaptic GluR abundance? In Drosophila and the rat nucleus accumbens, xCT is thought to modulate glutamatergic synapse strength by regulating the concentration of extracellular glutamate (Augustin et al., 2007; Grosjean et al., 2008; Scofield and Kalivas, 2014). This glutamate either directly desensitizes receptors (Featherstone and Shippy, 2008; Chen et al., 2009) or modulates mGluR activity to effect changes in synaptic strength (Scofield and Kalivas, 2014). In this chapter that either way xCT works via changes in extracellular glutamate. I will demonstrate that the decrease in ambient extracellular glutamate in xCT-/- compared to controls can be maintained in the slice. I will show that ambient extracellular glutamate is decreasing over time in control slices. I will show that mEPSC amplitudes in slice electrophysiology experiments are increasing over time and this is due to loss of extracellular glutamate.

6.2 xCT-/- mice have significantly less ambient extracellular glutamate compared to controls.

As predicted average ambient extracellular glutamate concentration is significantly decreased in slices from xCT-/- compared to controls (control = 5.328 ± 0.2746 μM N=5, xCT-/- = 2.401 ± 0.1523 μM N=2; p= 0.0015). This is a 54% decrease which is on par with the decrease observed by in vivo microdialysis measurements taken in the hippocampus (De Bundel et al., 2011).

One of the largest concerns initially with performing these experiments was that the decrease in ambient extracellular glutamate seen with loss of the xCT transporter in vivo could
not be maintained in slices. However, these experiments have shown that the decreased glutamate concentration in xCT-/- mice is maintained in hippocampal slices.
Figure 33: xCT-/− mice have significantly less ambient extracellular glutamate compared to controls. Bar graph depicting average ambient extracellular glutamate concentration. Glutamate is significantly decreased in slices of xCT-/− compared to control slices (control = 5.328 ± 0.2746 μM N=5, xCT-/− 2.401 ± 0.1523 μM N=2; p= 0.0015). This is a 54% decrease in ambient extracellular glutamate.

6.3 Ambient extracellular glutamate decreases in control slices over time.

The process of isolating hippocampal slices from the brain and continuously flushing the slices with aCSF led us to hypothesize that extracellular glutamate is percolating out of the slice
over time. Decreasing ambient extracellular glutamate would result in more functional receptors and increased mEPSC amplitudes over time. So mEPSC amplitude, as a direct measure of functional receptors, is inversely proportional to extracellular glutamate concentration.

Microdialysis measurements of glutamate were taken in hippocampal slices over time from when slices were patchable.

The microdialysis probe was placed at a depth of 20 μm directly at CA3-CA1 synapses or in a similar location to where immunohistochemical images were taken. Glutamate concentration measurements were taken every thirty minutes over the 6 hours window that electrophysiological recordings can be made. Thank you to Giovannie Ojeda-Torres and Dr. Scott Shippy for their collaboration which made these measurements possible. The results show that extracellular levels do appear to decline in slices over time in control slices (Figure 38).

As predicted a linear regression revealed a significant nonzero negative slope (Figure 38, slope= -0.01316 ± 0.004506 μM/min, linear regression p= 0.0049). Interestingly, xCT-/- mutants did not exhibit a significant nonzero slope over time (0.008544±0.002132 μM/min, linear regression p= 0.2265, N=7) which is consistent with the stable mEPSC amplitudes.

If we compare glutamate concentration measurements between 1-3 hours to samples taken between 3-6 hours, controls show a significant decrease in glutamate concentration, whereas xCT-/- mice do not (Fig. 8b; control: 1-3h = 6.675 ± 0.7199 μM, N=7; 3-6h = 4.205 ± 0.4454 μM, N=5; Student t-test p= 0.0193. xCT-/-: 1-3 hrs= 2.908 ± 0.04800 μM, N=2; 3-6hrs= 1.979 ± 0.2392 μM, N=2; Student t-test p= 0.0626).

We predicted that the increased glutamate initially after slicing would be largely due to the process of slicing. Isolation of the hippocampus would damage cells and the blood- brain barrier
which would release intracellular stores of glutamate. However, because the xCT-/- slices do not seem to exhibit a nonzero downward slope perhaps the initial increase in ambient extracellular glutamate is actually due to the xCT transporter. As previously discussed the transporter is activated by ROS, oxygen, and lipopolysaccharide located on gram-negative bacteria. (Bannai, 1984; Bannai et al., 1989; Sato et al., 2002; Sato et al., 2005; Kigerl et al., 2012). It is reasonable to suspect that during the surgery and slice preparation any one or all of these activators might be present which could increase transporter activity. This does not explain, however, why the glutamate concentration is going down in controls and not xCT -/- mutants. One hypothesis for why glutamate is decreasing overtime is that initially the EAATs cannot keep up with the uptake of such high glutamate concentrations. Eventually they are able to get the high concentration under control. While in the xCT mutants the EAATs are able to consistently manage the lower concentration of glutamate overtime. Further analysis is required to determine their relative concentrations.
Figure 34: Ambient extracellular glutamate microdialysis concentration measurements from hippocampal slices over time. In control slices extracellular glutamate declines over time at a slope of 0.01316 ± 0.004506 μM/min (linear regression p = 0.0049). Mutant xCT-/- did not exhibit a nonzero slope overtime. An ANOVA was performed and on average across all time points xCT-/- exhibited lower glutamate concentrations than controls.
Figure 35: Glutamate concentration in slices at 1-3 hours vs 3-6 hours in xCT-/- compared to control mice. Bar graph comparison of glutamate concentrations taken between 1-3 hours compared to concentration measurements taken between 3-6 hours, controls show a significant decrease in glutamate concentration (Mean ±SEM control: 1-3h = 6.675 ± 0.7199 μM, n=7; 3-6h = 4.205 ± 0.4454 μM, N=5; Student t-test p= 0.0193); whereas xCT-/- mice do not (xCT-/- 1-3 hrs= 2.908 ± 0.04800 μM, N=2, 3-6hrs= 1.979 ± 0.2392 μM; Student t-test p= 0.0626).

6.4 Control mEPSC amplitudes phenocopy xCT-/- over the time due to decreased glutamate.
If the increased xCT -/- mutant mouse synapse strength that we measured here is due to reduced extracellular glutamate, then it should also occur in control mice over time, as extracellular glutamate is washed from the slice by artificial CSF, which does not normally contain glutamate. Extended (up to 6 hours) incubation of hippocampal slices in aCSF is not uncommon, but changes over time have not been systematically examined. We therefore measured mEPSC amplitudes in control and xCT -/- mutant hippocampal slices, after varying durations in glutamate-free aCSF. Fig. 37 summarizes our results. Consistent with the idea that control mEPSCs are suppressed by extracellular glutamate, mEPSC amplitudes were significantly increased as slices were incubated in glutamate-free aCSF before recording (Fig. 37a; 0.02259 ± 0.003837 pA/min; Linear regression, p = 0.0002). In contrast, xCT -/- mutant mEPSCs did not significantly increase under the same conditions (Fig. 37a; 0.006357 ± 0.008395 pA/min; Linear regression p= 0.4664).

If we compare mEPSC amplitudes recorded between 1-3 hours to recordings taken between 3-6 hours, controls show a significant increase in mEPSC amplitudes, whereas xCT -/- mice do not (Fig. 37b; control 1-3h = 6.122± 0.4397 pA, n=7; 3-6h = 10.07 ± 1.144 pA, n=5; Student t-test p=0.0045; xCT -/- 1-3h = 9.892 ± 0.9401 pA, n=8; xCT -/- 3-6h = 11.31 ± 1.712 pA, n=4; Student t-test p= 0.4462) Furthermore, the increase in control mEPSC amplitudes over 6 hours were almost identical to the differences between control and xCT -/- mEPSCs recorded within the first 3 hours. These data are consistent with the idea that xCT suppresses mEPSC amplitudes in control mice by maintaining high levels of extracellular glutamate.

The decrease in extracellular glutamate and the increase in mEPSC amplitude overtime do demonstrate that there are experimental changes in slice electrophysiology over the time and this should be taken into consideration when planning any slice electrophysiology experiments.
Figure 36: Control mEPSC amplitudes phenocopy xCT-/ mutant mEPSCs as glutamate is percolating out of the slice over time. (a) In control recordings mEPSC amplitudes (black) were significantly increased or had a significant non-zero slope as slices were incubated in glutamate-free aCSF before recording (Fig. 8a; slope = 0.02259 ± 0.003837 pA/min; Linear regression, p = 0.0002). In contrast, xCT -/- mutant mEPSCs (red) did not significantly increase under the same conditions (Fig. 8a; slope= 0.006357 ± 0.008395 pA/min; Linear regression p= 0.4664). (b) Box-and-whisker plot comparison of mEPSC amplitudes recorded between 1-3 hours to recordings taken between 3-6 hours, controls show a significant increase in mEPSC amplitudes, whereas xCT -/- mice do not (Mean (+ sign) ±SEM control 1-3h = 6.122± 0.4397 pA, n=7; 3-6h = 10.07 ± 1.144 pA, n=5; Student t-test p=0.0045; xCT -/- 1-3h = 9.892 ± 0.9401 pA, n=8; xCT -/- 3-6h = 11.31 ± 1.712 pA, n=4; Student t-test p= 0.4462).
CHAPTER VII: DISCUSSION

7.1 Chapter Summary

This chapter will describe System Xc’s role in human disease. I will then discuss the implications this analysis has within the context of the xCT transporter field. I will then describe
areas within the field that require future experiments and future experiments that build upon the
analysis described here.

7.2 System Xₖ⁻ misregulation in human disease

7.2.1 The xCT transporter and schizophrenia

Functional magnetic resonance studies in the prefrontal cortex have implicated schizophrenia as being a hypoglutamatergic state (Barch et al., 2001). Phencyclidine (PCP), a noncompetitive NMDA antagonist, causes schizophrenic-like symptoms in rats and humans and worsens symptoms of schizophrenia in patients. Due to these effects it is often used as a model for schizophrenia (Krystal et al., 1994; Khurgel et al., 1996; Malhotra et al., 1997; Abi-Saab et al., 1998; Gaskin et al., 2014)

PCP elevates extracellular glutamate levels in the prefrontal cortex and this effect could be blocked by N-acetylcystine an xCT transporter prodrug. PCP also results in deficits in working memory and this could be blocked by N-acetylcystine pretreatment. The reversal of deficits in working memory could be blocked by CPG or a blocker of System Xₖ⁻ (Baker et al., 2008).

PCP and schizophrenia are also linked to disruption in sensory motor gating or the ability of sensory information to influence behavior. Sensory motor gating deficits could be reversed with the infusion of N-acetylcystine which is thought to increase xCT activity. This reversal could be inhibited by the co-infusion of CPG (Lutgen et al., 2013).
Postmortem tissue from the prefrontal cortex of human schizophrenic patients indicates a significant increase in the xCT subunit in the dorsolateral prefrontal cortex (Baker et al., 2008). In contrast, schizophrenic patient’s exhibit decreased glutathione seen in the medial prefrontal which could be consistent with decreased xCT transporter activity. However, it does seem to suggest altered nonsynaptic glutamate dynamics whether this is due to an increase or decrease in glutamate seems to remain unclear (Do et al., 2000).

7.2.2 The xCT transporter and gliomas

Cystine-glutamate misregulation has also been implicated in glioma brain tumors. In glioma cell lines glutamate uptake by GLT-1 (or EAAT-2) in astrocytes is highly reduced. This is due to a reduction in GLT-1 and a mislocalization of GLAST to the cell’s nucleus rather than the plasma membrane. Cystine-glutamate exchange is in contrast enhanced in these cell lines. Indicating that glioma cells are releasing larger than normal quantities of extracellular glutamate with less reuptake which could contribute to the excitotoxic neuronal cell death, necrosis and seizures associated with brain tumors (Ye et al., 1999).

The increase in xCT mediated glutamate release results in increased cystine uptake in gliomas. This cystine is quickly reduced to cysteine which creates more glutathione. Therefore, gliomas not only release excitotoxic levels of glutamate but also have increased antioxidant protection from reactive oxygen species (ROS). The increased ROS protection protects gliomas from caspase- mediated apoptosis. Sulfazalazine, an xCT inhibitor, was able to reduce the increase in glutathione and slow the growth of tumors. (Chung et al., 2005a)
7.2.3 The xCT transporter and addiction

During withdrawal from repeated cue-induced cocaine administration ambient extracellular glutamate concentration is significantly reduced in the nucleus accumbens. This decreased ambient extracellular glutamate could be restored with infusion of NAC which is thought to increase xCT transporter activity. Reverse microdialysis of CPG, an xCT inhibitor, into the nucleus accumbens could reduce extracellular glutamate to levels observed in cocaine withdrawal in control rats. The addition of CPG into the nucleus accumbens of cocaine withdrawing did not induce further decreased ambient glutamate indicating that CPG mimics the effect of reduced ambient extracellular glutamate during cocaine withdrawal. Reinstatement of drug-seeking in cocaine withdrawing mice could also be decreased with the infusion of NAC (Baker et al., 2003).

During cocaine withdrawal both cystine-glutamate exchange and GLT-1 or EAAT-2 have been implicated in reduced extracellular glutamate. Also, the return of extracellular glutamate to control levels with NAC treatment has been thought to be the result of restoration of cystine-glutamate or GLT-1 exchange. Recently, Reissner et. al. (2014) has shown that NAC effects GLT-1 not xCT transporter exchange when reducing cue-induced cocaine reinstatement (Reissner et al., 2014). These experiments could suggest that NAC may not work on the xCT transporter at all and alters extracellular glutamate by affecting the EAATs. Further experiments are required to make this determination.

7.3 Discussion

We have shown that loss of xCT leads to accumulation of postsynaptic glutamate receptors in CA3-CA1 synapses, with dramatic functional consequences, and that these changes are likely
due to alterations in ambient extracellular glutamate. A similar mechanism for synapse modulation by glial xCT was previously demonstrated in *Drosophila* (Augustin et al., 2007; Grosjean et al., 2008), and suggested by pharmacological studies in rats (Kalivas, 2009; Scofield and Kalivas, 2014).

Our results are physiologically significant. Loss of xCT triggered synaptic changes comparable to forms of plasticity typically associated with behavioral changes and learning, such as long-term potentiation (LTP) (Bliss and Lomo, 1973; Dunwiddie and Lynch, 1978). Thus, it is conceivable that changes in xCT activity might mediate some forms of learning or other changes in behavior. Most studies of xCT expression and activity have used cultured cells and treatments designed to understand the role of xCT during pathological oxidative stress as oxygen itself is a potent modulator of xCT. (Bannai et al., 1989; Lewerenz et al., 2012). However, pharmacological manipulation of mGluR 2/3, protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent kinase II (CamKII) also affect xCT activity in rat cortical astrocytes (Tang and Kalivas, 2003). It is likely that xCT activity is also regulated by extracellular cystine, as well as lactic acid which appears to inhibit xCT activity in cortical astrocytes (Koyama et al., 2000). Future studies will need to explore the regulation of xCT in further detail, so that we can better understand the conditions under which xCT might in turn regulate synaptic strength, as demonstrated in the present study.

If regulation of xCT is an important determinant of synaptic strength, then the misregulation of xCT could be linked to neurollogical disease. Consistent with this notion, drug craving behavior in humans and animals is associated with increased transmission from the prefrontal cortex to the nucleus accumbens, a region which is also modulated by input from the hippocampal CA1 cells described in our study. As an integration center for responses to various
stimuli, the nucleus accumbens is therefore an ideal location for behaviorally-relevant modulation by glial xCT. Consistent with this idea, withdrawal from repeated self-administered cocaine in rats leads to reduced nucleus accumbens xCT expression and activity, with an accompanying significant decrease in extracellular glutamate (Baker et al., 2003; Knackstedt et al., 2010). In further support of the idea that loss of xCT activity leads to changes that promote subsequent drug-seeking behavior, N-acetylcysteine (which promotes xCT activity) reduces drug-seeking (Kau et al., 2008) and increases diminished extracellular glutamate levels in the nucleus accumbens during cocaine withdrawal. Direct administration of cystine, xCT’s major substrate, also increases extracellular glutamate in cocaine-withdrawn rats. Conversely, administration of CPG, an xCT inhibitor, into the nucleus accumbens in cocaine-withdrawn rats did not have an effect on extracellular glutamate presumably because xCT already has decreased activity (Baker et al., 2003). Along similar lines, rats that self-administered nicotine and were withdrawn from nicotine also showed reduced xCT expression in the nucleus accumbens, and human smokers treated with N-acetylcysteine reported a decrease in cigarettes smoked (Knackstedt et al., 2009).

Altered glutamatergic transmission within the dorsolateral prefrontal cortex also likely contributes to the symptoms of schizophrenia (Bunney and Bunney, 2000), and xCT may be involved here too. Postmortem analysis of xCT expression in the prefrontal cortex of human schizophrenic patients reveals a slight but significant increase in xCT levels (Baker et al., 2008). Furthermore, N-acetylcysteine reduces the psychomimetic effects of phencyclidine (PCP; a pharmacological model of schizophrenia) in rats (Baker et al., 2008; Chen et al., 2010) and improves symptoms of schizophrenia and bipolar disorder in humans (Berk et al., 2008b; Berk et al., 2008a; Lavoie et al., 2008; Bulut et al., 2009).
Most neuroscientists, when they think of extracellular glutamate, associate it with excitotoxicity. Apparently, glutamate uptake transporter (EAAT) activity is normally sufficient to prevent runaway xCT activity from causing cell death. In some cases, however, xCT activity is upregulated while EAAT activity is reduced. One such case is that of gliomas which, through simultaneous upregulation of xCT and reduced EAAT, appear to excitotoxically 'clear space' for their own growth and migration (Ye et al., 1999; Ye and Sontheimer, 1999; Takano et al., 2001; Chung et al., 2005b; Robert and Sontheimer, 2013). An understanding of xCT function is critical for development of effective treatments targeting this mechanism.

Our experiments revealed that, under normal conditions, xCT suppresses glutamatergic synaptic strength. This conclusion is consistent with data from Drosophila and inferences from pharmacological studies in rats (Baker et al., 2002; Baker et al., 2003; Augustin et al., 2007; Grosjean et al., 2008; Scofield and Kalivas, 2014). But what exactly is the mechanism by which xCT might suppress synapse strength?

In Drosophila, ambient extracellular glutamate, mostly attributable to the xCT homolog, genderblind, works directly on postsynaptic glutamate receptors to trigger their removal from the synapse (Augustin et al., 2007; Chen et al., 2008; Piyankarage et al., 2008). In rats, several lines of evidence suggest that xCT-secreted glutamate tonically activates group II mGluRs on the presynaptic terminals of prefrontal cortex neurons forming synapses into the core of the nucleus accumbens (Xi et al., 2002; Moussawi and Kalivas, 2010; Scofield and Kalivas, 2014). This in turn is thought to suppress synaptic transmission by activation of presynaptic potassium channels and inhibition of presynaptic calcium channels (Moussawi and Kalivas, 2010; Scofield and Kalivas, 2014). Thus, in Drosophila the xCT homolog genderblind controls synapse strength arises through glutamate binding, and postsynaptic receptor desensitization leading to removal of
glutamate receptors from the synapse. While in rats, the reduction in synapse strength is thought to be due predominantly to presynaptic inhibition.

The reduction in hippocampal CA3-CA1 synaptic transmission that we observed might be due to a mix of the mechanisms described in *Drosophila* and rats. Our experiments argue strongly that the changes in CA3-CA1 synapse strength in xCT -/- mutant mice are due mostly, if not entirely, to changes in postsynaptic glutamate receptor abundance. This is consistent with changes observed in genderblind mutant *Drosophila* (Augustin et al., 2007), and along the same lines as the increase in AMPA receptors observed in the nucleus accumbens of cocaine-withdrawn rats (Boudreau et al., 2007; Wolf and Ferrario, 2010). To account for the rat pharmacology implicating mGluR involvement, we note that CA3-CA1 synapses also display mGluR-dependent long-term-depression (LTD) (Oliet et al., 1997; Overstreet et al., 1997; Palmer et al., 1997). mGluR-dependent LTD in hippocampal CA1 neurons occurs when activation of postsynaptic Group I mGluRs by extrasynaptic glutamate triggers a series of intracellular signaling events leading to reduced postsynaptic glutamate receptor expression and altered trafficking (Gladding et al., 2009). If xCT activity *in vivo* contributes to constitutive LTD in CA1 neurons, loss of xCT would be expected to cause increased postsynaptic glutamate receptor abundance exactly as we observed. In this case, modulation of synapse strength by xCT is an example of glia regulating LTD. Future experiments will be directed at exploring this possibility.

**7.4 Future Directions**

There are several details of the xCT transporter and ambient extracellular glutamate regulation that require further exploration to understand its implication for brain function and for
developing therapeutics for human disease. Some of these future directions described below will be examined in detail in the Featherstone laboratory whereas others are outside of the scope of our experimental expertise.

Firstly, the xCT transporter in Drosophila was localized to a previously undefined subset of glia (Augustin et al., 2007). In mammals xCT transporter protein has been localized largely to astrocytes, microglia, Müller cells, glioma cells, and immature cortical neurons. Suprisingly, mature neurons appear to exhibit little or no xCT activity (Zerangue and Kavanaugh, 1996; McBean and Flynn, 2001; Pow, 2001; Chen and Swanson, 2003; Verrey, 2003). Antibodies for the xCT transporter have been difficult to engineer for use in brain slices because the xCT transporter is a highly lipophilic protein with 12 transmembrane domains (Mastroberardino et al., 1998; Bassi et al., 2001; Verrey, 2003; Verrey et al., 2004). The use of specific xCT antibodies in western blots works well, and in both the xCT/- and sut/sut mice lines the protein is completely lost (McCullagh and Featherstone, 2014). There is also no evidence of mRNA transcripts for xCT/- mutants in northern blots (Chintala et al., 2005; Sato et al., 2005). Experiments with an antibody against α-aminoapidate a (substrate of the xCT transporter) found accumulation to be largely restricted to glia and this has led to the conclusion that the transporter is probably located on glia (Pow, 2001). α- aminoapidate, however, has long been known as a specific gliotoxin and this gliotoxicity has been attributed to loss of function of system X_c^-.

However, α-aminoapidate is also a substrate for system X^-AG transporters or the EAATs which are responsible for Na^+-dependent glutamate uptake. (Khurgel et al., 1996; Tsai et al., 1996). A specific antibody for the xCT transporter that can work immunohistochemically in brain slices is crucial for determining if the transporter is actually located on glia.
System Xc⁻ transports glutamate and it only diverges from the GluR2 subunit of the AMPA receptor by having a lipophilic binding domain (Bridges 2012). This has made specific inhibitors and activators difficult to create. For example the commonly employed inhibitor CPG is sold as a metabotropic glutamate receptor inhibitor and Homocysteic acid is a known NMDA receptor agonist (Olney et al., 1987). Kalivas (2014) had recently shown that N-acetylcystine, a commonly employed xCT agonist, might actually instead inhibit GLT-1 or EAAT-2 rather than activating xCT (Reissner et al., 2014). We have avoided nonspecific modulators in this analysis by employing mutant xCT-/− mice. However, if the transporter can be specifically modulated this would not only be important for further experimentation but it could also be an important target for drug discovery. I have shown that loss of extracellular glutamate can modulate postsynaptic receptors and synaptic transmission. Therefore, modulating the transporter can modulate extracellular glutamate which can cause significant changes in synaptic transmission.

Our lab and others have gone to great lengths to target a behavioral phenotype in xCT-/− mutants; however, the results remain unclear (McCullagh and Featherstone, 2014). For example DeBundel has published results showing that xCT-/− mice exhibit spatial working memory deficits (De Bundel et al., 2011). Our lab could not replicate these deficits in xCT-/−, however, we did see spatial working memory deficits in sut/sut mice (McCullagh and Featherstone, 2014). The xCT -/- mutant mice have been shown to exhibit impaired fear and passive avoidance memory, both of which are hippocampus-dependent learning paradigms (Li et al., 2012). Incredibly, Drosophila genderblind males try to mate and copulate with other males and this implicates the xCT transporter as being crucial to fundamental behavior. The behavioral phenotype is not only profound but can be turned on and off with heat inducible RNAi, and by altering glutamatergic synapse strength. The genderblind phenotype is attributable to increased
glutamatergic synaptic transmission and by feeding mutants gamma-D-glutamylglycine a competitive glutamate receptor the phenotype could be abolished (Grosjean et al., 2008). The field of synaptic transmission is based on the assumption that synaptic function is what controls behavior. The changes in glutamatergic synaptic strength shown in this analysis are on par with observations in genderblind flies. It is therefore somehow unsatisfying that there is not a profound behavioral phenotype in these mice. Better behavioral tests of sexuality in mice need to be developed and further behavioral analysis needs to be employed.

This analysis has shown that there is a significant increase in the number of receptors at the synapse and this could be visualized by using immunohistochemistry for the GluR1 subunit and measuring the area. The postsynaptic MAGUK SAP-102 which was used in this analysis as a postsynaptic marker also exhibited an increase in puncta size. SAP-102 has been shown to cause clustering of AMPA receptors at immature synapses (Elias et al., 2006) and this is aligned with the results that we have demonstrated. However, this data could suggest more widespread changes in the size of the postsynaptic density, the morphology of dendritic spines, or even a merging of two dendritic spines. Either way it really highlights how impactful ambient extracellular glutamate is at the synapse. Further studies of dendritic morphology and electron microscopy could reveal the extent of postsynaptic remodeling due to loss of the xCT transporter and changes in ambient extracellular glutamate.

This analysis has focused on the AMPA receptor and its trafficking as a result of loss of xCT. It is hypothesized that at ambient glutamate concentrations nearly ¾ of all NMDA receptors will be desensitized, which is a larger fraction than AMPA receptors (Featherstone and Shippy, 2008). Further analysis is needed to determine if ambient extracellular glutamate has an effect on NMDA receptor trafficking.
I have shown that mEPSC amplitudes increase over time in control slices and this correlates with decreased extracellular glutamate. This analysis has brought about several yet unanswered questions, such as why is xCT glutamate not decreasing overtime? Is this because very little glutamate is actually diffusing out of the slice and the EAATs are maintaining the concentration of ambient extracellular glutamate? Also, we were surprised at how high the initial glutamate concentration was in the control slices. At concentrations this high we would expect that most AMPARs would be desensitized and functionally unavailable. If there are only very few functionally available receptors then how can we make electrophysiological recordings. Interestingly, for control mEPSC recordings I have only taken one recording prior to 60 minutes. Whereas for the xCT-/- mice I took five recordings within that time period. Furthermore, as previously discussed there is a question as to whether the initial high concentration of extracellular glutamate is actually due to the xCT transporter responding to the slicing conditions. Our lab has plans to explore this further.

Lastly, one hypothesis in the field suggests that ambient extracellular glutamate, released via system xCT, activates mGlu receptors which inhibit presynaptic vesicular release and this decreases synaptic transmission (Baker et al., 2002; Baker et al., 2003; Reissner et al., 2014; Scofield and Kalivas, 2014). Our analysis has shown that the xCT transporter suppresses the number of postsynaptic receptors which causes a large decrease in synaptic transmission. In Drosophila this requires glutamate binding to ionotropic receptors (Chen et al., 2009). Does the mechanism in mice work like Drosophila or is it a form of mGlu receptor LTD?
CHAPTER 8: GENERAL SUMMARY

Work in thesis has brought new methods to the field of neuroscience. The quantitative immunohistochemistry performed has pushed the boundaries of a commonly employed technique in brain slices. Prior to this analysis very little quantitative immunohistochemistry
analysis showing changes in number of receptors had performed done in slices using a confocal microscope. In *Drosophila* at the neuromuscular junction similar methods are regularly performed. This new slice immunohistochemical method can actually be used to quantify changes in number of receptors at the synapse. It can also distinguish between whether the change in receptors is due to receptor insertion, resulting in changes in area, or due to changes in the density of receptors via an intensity measurement. It has also provided a novel method to quantify changes in synapse number and even show changes in the size of the postsynaptic density or the postsynaptic scaffolding proteins.

Glutamate measurements in hippocampal slices are also a novel method brought to the field of neuroscience by this analysis. Prior to this analysis it was not clear if extracellular glutamate could be measured in brain slices to the precision required to observe changes in concentration. This method indeed does demonstrate that changes in glutamate concentration can be observed overtime and can be observed when genetically altering a potent modulator of its concentration.

This analysis has also brought new methods to the Featherstone lab. Prior to these experiments the Featherstone lab has largely used *Drosophila* electrophysiology, immunohistochemistry, and molecular techniques. This analysis has now brought slice electrophysiology in mice to the lab as a tool for analysis and for future experiments. The introduction of slice electrophysiology required extensive research and collaboration with other labs to create the conditions and patch rig required for slice electrophysiology. It should be acknowledged that these collaborators include Dr. Simon Alford and Dr. Christian Hansel and their students. Thanks to both of you for your help and expertise.
The work in this thesis is important to the field of neuroscience and the body of knowledge for several reasons: 1) much of the xCT field assumes loss of the xCT transporter results in an increase in synapse strength in mice, however, this has not been fully demonstrated until this analysis. In fact this is the only electrophysiological analysis that has been performed to this precision using the whole-cell patch clamp technique on rodents in this field.

2) This analysis has demonstrated that loss of the xCT transporter results in an increase in the number of postsynaptic receptors which is in contrast to the assumption described by Peter Kalivas that the increase in synapse strength is due to an increase in presynaptic release. Under normal conditions the transporter does not induce a form of presynaptic inhibition but suppresses the number of postsynaptic receptors.

3) This analysis shows a mechanism where an increase in the number of postsynaptic receptors can cause significant changes in synapse strength.

4) The magnitude of the modulation of synapse strength observed here is a novel mechanism on par with the sizes observed in LTP and LTD which are considered to be the hallmarks of synaptic plasticity.

5) This analysis demonstrates that the glutamate released by the xCT transporter is actually what causes the suppression of postsynaptic receptors and synaptic strength. Previously, it has only been assumed that because the transporter releases glutamate that changes in synaptic strength must be due to the actual glutamate.

6) If the transporter is actually located on glia, this work has highlighted a real and novel mechanism where glia can modulate synaptic transmission and induce synaptic plasticity on par with strong inducers of LTP and LTD. This also highlights the deficit in proper antibodies for the
transporter, which could confirm immunohistochemically where the transporter is actually located.

7) This analysis demonstrates a highly conserved mechanism that was first demonstrated in *Drosophila* and now has been shown to work similarly in mice. This highlights how studies of lower order animal models can truly be a fruitful starting point for understanding CNS function and cellular mechanisms.

8) This transporter has been implicated in several human diseases including addiction, gliomas, and schizophrenia. This makes the transporter a novel target for drug therapeutics and stresses the importance of finding more specific activators and inhibitors of the transporter. This analysis suggests that if we can specifically modulate the transporter’s activity we can powerfully modulate synaptic transmission. If we can modulate synaptic transmission then we can modulate behavior and disease.

*Thank you,*

*Leena*
APPENDIX: Animal Care Committee Approvals
3/16/2011

David Featherstone
Biological Sciences
M/C 067

Dear Dr. Featherstone:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and renewed on 3/16/2011.

**Title of Application:** Immunohistochemical Analysis of Proteins in the Mouse Brain

**ACC NO:** 10-025

**Original Protocol Approval:** 3/16/2010 (3 year approval with annual continuation required).

**Current Approval Period:** 3/16/2011 to 3/16/2012

Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol.

**UIC is the only performance site approved for this protocol.**

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

[Signature]

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/kg
cc: BRL, ACC File
2/16/2011

David Featherstone
Biological Sciences
M/C 067

Dear Dr. Featherstone:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and renewed on 2/16/2011.

Title of Application: Modulation of Mouse Synapses
ACC NO: 10-003

Original Protocol Approval: 2/16/2010 (3 year approval with annual continuation required).

Current Approval Period: 2/16/2011 to 2/16/2012

Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol.

UIC is the only performance site approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/kg
cc: ACC File
December 19, 2012

David Featherstone
Biological Sciences
M/C 067

Dear Dr. Featherstone:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 12/18/2012. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: Modulation of Mouse Synapses

ACC Number: 12-218

Initial Approval Period: 12/18/2012 to 12/18/2013

Current Funding: Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. **UIC is the only performance site currently approved for this protocol.**

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW’s “What Investigators Need to Know about the Use of Animals” (http://grants.nih.gov/grants/olaw/InvestigatorsNeedtKnow.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/ss
cc: BRL, ACC File


Gaskin PL, Alexander SP, Fone KC (2014) Neonatal phencyclidine administration and post-weaning social isolation as a dual-hit model of 'schizophrenia-like' behaviour in the rat. Psychopharmacology (Berl).


Wolf ME, Ferrario CR (2010) AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. Neuroscience and biobehavioral reviews 35:185-211.


VITA

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- Accomplished whole-cell patch clamp electrophysiologist in brain slices.
- Adept at quantitative confocal microscopy & immunohistochemistry.
- Transformed my electrophysiology rig from fly patching to patching in mouse brain slices.
Teaching Assistant 2010 to present  University of Illinois at Chicago

- The position functions to teach a Junior and Senior level undergraduate genetics laboratory, microbiology laboratory and microbiology lecture.
- Duties involve lecturing and facilitating the students in performing genetics related experiments.
- I administer all tests, quizzes, assign projects, and presentations.
- I perform all grading and maintain all grading records.
- Act as a mentor and advisor.

Clinical Research Specialist 2008 to 2010  University of Illinois at Chicago

- The position functions to coordinate and assist in research activities as they relate to the Department of Neurosurgery.
- A part of multi-disciplinary team in the care of subjects receiving treatment on clinical trials and to give surveys for data collection purposes.
- Responsible for coordinating subject study visits, hosting monitoring visits, facilitating research audits, trained in good clinical practice and regulatory compliance, and trained to prepare Institutional Review Board (IRB) submissions.
- Prepared for and participated in a Food and Drug Administration audit for a Sponsor-Investigator that holds an Investigational Device Exemption.

Microbiologist 2006 to 2008  Hospira, Inc.
Pharmaceuticals

- Member of the Biological Quality Department, specializing in bacterial endotoxin testing.
- Our group plays a vital role in the assurance of product quality in all stages of manufacturing from development to distribution.

Unit Secretary 2002 to 2005  The Cleveland Clinic

- Acquired extensive knowledge about the inner workings of a fast paced, surgical nursing unit.
- Transcribed doctor’s orders, including medications, dietary instructions, physical limitations, and testing.
- Expected to recognize and follow applicable hospital procedures for critical patient emergencies.
Laboratory Technologist 2003 to 2004  
Loyola University Chicago

- Duties included isolating the cytoskeleton protein spectrin from erythrocytes.
- I acquired training in spectrophotometry, Western blots, gel electrophoresis, affinity column chromatography, thin layer chromatography, dialysis, and distillation.
- Expected to respond to the needs of graduate students performing research in the laboratory.

Primary Publications:

Williams LE, Featherstone DE. Regulation of mouse hippocampal synaptic transmission by glial xCT. Journal of Neuroscience. Sent out for review.


Awards and Achievements:

Liberal Arts & Sciences PhD travel award (University of Illinois at Chicago; 2013).

Biological Quality MVP Award for Exceptional Efforts in the category of Outstanding Performance (Hospira Inc. Pharmaceuticals; 2007).

Biological Quality MVP Award for Exceptional Efforts in the category of Honorable Mention/Outstanding Performance (Hospira Inc. Pharmaceuticals; 2006).

**Mulcahy Scholarship** (Loyola University Chicago, The College of Arts and Sciences Dean’s Office; 2004).

**Research Activities:**

**Participation on Animal Care and Use- Approved Animal Research Protocols**

- Immunohistochemical and electrophysiological analysis of proteins in the mouse brain (Doctoral Student, University of Illinois at Chicago; 2010)

**Participation on Institutional Review Board (IRB)- Approved Clinical Research Protocols**

- A Prospective, Multi-Center Trial of NovoTTF-100A Compared to Best Standard of Care in Patients with Progressive of Recurrent GBM (Clinical Study Coordinator, University of Illinois at Chicago; 2008)

- Implantable Systems Registry Study (Clinical Study Coordinator, University of Illinois at Chicago; 2008)

- An Observational, Prospective Study of Duragen vs. Duraguard in Chiari Surgery (Clinical Study Coordinator, University of Illinois at Chicago; 2008)

- Use of a Occipital Nerve Neurostimulator in the Treatment of Chronic Migraine Headaches (Clinical Study Coordinator, University of Illinois at Chicago; 2008)

- Cervical Spinal Cord Stimulation for Prevention of Cerebral Vasospasm (Clinical Study Coordinator, University of Illinois at Chicago; 2008)

**Research Funding**

A Prospective, Multi-Center Trial of NovoTTF-100A,

(Clinical Research Coordinator, University of Illinois at Chicago & NovoCure 2008-2010)

Studying the Enzyme Activity of Glutathione Reductase in an Aging Population of Thirteen-lined Ground Squirrels,*Spermophilus tridecemlineatus*

(Undergraduate Researcher, Mulcahy Scholars Program, Loyola University Chicago; 2004)
Presentations:

*Elucidating the Function of Ambient Glutamate & xCT at the Mouse Synapse* Williams LE, Feathertone DE. Poster Presentation at the Society for Neuroscience Annual Meeting (November 10, 2013; San Deigo, California, U.S.A).

Williams L.E. *Elucidating the Function of Ambient Glutamate & xCT at the Mouse Synapse*. Brain Bee University of Illinois at Chicago

*Elucidating the Function of Ambient Glutamate & xCT at the Mouse Synapse*. Preliminary Examination, University of Illinois at Chicago (2013)


Continuing Education Activities:

**Chicago Society for Neuroscience.** (Northwestern University 2013)

**Achieving Excellence in Clinical Research: Scientific, Ethical and Operational Considerations.** (McDonald Hamburger University- Advocate Healthcare 2009)

**2009 ACRP Clinical Research Educational Conference.** (Northwestern University, 2009)

**Neurovascular Symposium 2008.** (University of Illinois at Chicago, Department of Neurosurgery; 2008)

**CRC Basic Training: A Practical Introduction to the Clinical Research Coordinator Role**

(Northwestern University Clinical & Translational Sciences Institute, Feinberg School of Medicine; 2008)
Collaborative IRB Training Initiative (University of Miami; 2008)

HIPAA Research 101 Requirements(University of Illinois at Chicago, 2008)

Memberships:

Member Society for Neuroscience (2013-present)

International:

Loyola University John Felice Rome Center