Behavioral Characterization of System xc- Mutant Mice

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THESIS

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To all of the mice who gave their lives for my research.

But, Mousie, thou art no thy lane [*you aren't alone*]
In proving foresight may be vain:
The best laid schemes o' mice an' men
Gang aft a-gley, [*often go awry*]
An' lea'e us nought but grief an' pain,
For promised joy.

Excerpted from Robert Burn's Poem *To a Mouse*, 1786

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LIST OF ABBREVIATIONS

3' RACE- Rapid Amplification of cDNA Ends

AAREs- amino acid response elements

ALS- amytrophic lateral sclerosis model

AMPA- alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

B6- C57/BL6 background mouse strain

Ca- calcium

(S)-4-CPG- (S)-4-carboxyphenylglycine

CSF- cerebral spinal fluid

DIDS- 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid

EAAT- excitatory amino acid transporter

ENT-1- the equilabrative nucleotide transporter

gb- *genderblind* the system xc- homolog in Drosophila

GSH- glutathione

HATs- heteromeric amino acid transporters

iGluR- ionotropic glutamate receptor

I.P.- intraperitoneal

K- potassium

kDa- kilo Dalton

LTP- long-term potentiation

M- molar

mGluR- metabotropic glutamate receptor

mRNA- messenger RNA

NAC- *N*-acetylcysteine

Na- sodium

NMDA- N-methyl-D-aspartate

PCR- polymerase chain reaction

S-CPG- S-4-Carboxyphenylglycine

SAS- sulfasalazine

SnJ- C3H/He/SnJ

sut- subtle gray xCT mutant mouse

t-BHQ- *tert*-butylhydroquinone

TBOA- threo-beta-Benzyloxyaspartate

xCT- cystine glutamate transporter/xCT-/- mutant mouse strain

µL- microliter

SUMMARY

System xc- is a sodium-independent cystine-glutamate exchanger, taking in one cystine and extruding a glutamate molecule. This transporter may be important in maintaining the balance of extracellular glutamate levels and internal levels of cystine. As a consequence, system xc- has been shown to regulate glutamate receptor function (both iGluRs and mGluRs) through control of extracellular glutamate levels and intracellular glutathione synthesis through cystine import. Thus, we hypothesize that impairments in this transporter may lead to changes in behavior. To test whether system xc- regulates behavior, I chose to examine system xc- mutant mice. Specifically I examined behavior in two different knockouts of the xCT gene (*sut* and *xCT*) and a cross of the two strains (*xCT/sut*) and their respective genetic controls (*C3H/He/SnJ, C57BL/6J, B6/SnJ*).

There was no consistent behavioral phenotype across the two strains of system xc- knockout, or the cross, compared to their controls. Also, male and female *xCT/sut* mice do not have reduced glutamate levels in the striatum or cerebellum. Homozygous *sut* mice do not have a reduction in glutamate levels in the cerebellum. Using Western blotting, all three strains of knockout are lacking expression of the xCT protein and do not compensate for glutamate levels by a change in expression of the excitatory amino acid transporters (EAATs). Female mice were also tested in these behavioral tasks and these behaviors are not regulated by changes in the estrus cycle. Therefore it seems that if system xc-

regulates behavior it is in a very subtle fashion that is difficult to see with the

techniques used in these experiments.

1 INTRODUCTION

Glutamate is the most abundant neurotransmitter in the nervous system.
The primary mechanism by which glutamate signaling occurs is through
synapses, however there is also an ambient level of extracellular glutamate that
may convey information to cells (Bergles et al., 1999). Extracellular glutamate
has been shown to have important roles in many behaviors, such as learning and
memory, and has been shown to be important in many diseases, particularly
depression, schizophrenia, epilepsy, addiction, multiple sclerosis and cancer
among others (Chung et al., 2005; Moran, 2005; Baker et al., 2007; Kristiansen
et al., 2007; Kalivas, 2009; De Bundel et al., 2011; Pampliega et al., 2011;
Sanacora et al., 2012). It is thought that cystine-glutamate transporters might
account for most of this extracellular glutamate release. *The current study aims
to test whether cystine-glutamate transport has effects on behavior.*

1.1 What are cystine-glutamate transporters?

The cystine-glutamate transporter (hereafter referred to as system xc-)
has been shown to transport cystine intracellularly while exporting glutamate in a
1:1 ratio (Bannai, 1986). This transport is sodium-independent utilizing the
concentration gradient of the two molecules. Glutamate is kept at low
concentrations extracellularly through actions of excitatory amino acid

transporters (EAATs) that remove glutamate from the extracellular space. Extracellular cystine levels are higher than intracellular levels because cystine is quickly reduced to cysteine to synthesize glutathione (GSH) inside the cell (Bassi et al., 2001). This reaction is chloride-dependent, cystine (a neutral molecule) is transported internally with a chloride molecule while glutamate (an anion) is exported keeping the transport electrochemically neutral (Gochenauer and Robinson, 2001).

Cystine-glutamate transporters are made up of a 12 transmembrane domain light chain (xCT)(Figure 1) and a heavy chain known as 4F2hc that has a single transmembrane domain (Sato et al., 1999; Bassi et al., 2001). The xCT protein confers the specificity of the transporter whereas 4f2hc is common to many other transporters (Sato et al., 1999; Bassi et al., 2001). The N and the C-terminal of the xCT protein are located inside the cell and the two subunits, (xCT and 4F2hc) are connected via a disulfide bridge similar to many other heteromeric amino acid transporters (HATs) (Wagner et al., 2001; Gasol et al., 2004). 4Fh2c is thought to be necessary for trafficking xCT to the plasma membrane (Bassi et al., 2001; Wagner et al., 2001). The majority of the research on system xc- has focused on *in-vitro* studies and the import of cystine through this transporter since it is a rate-limiting step for glutathione synthesis and important in oxidative stress (Figure 2) (Shih and Murphy, 2001; Shih et al., 2003).

Figure 1. Diagram of xCT protein with its twelve transmembrane domains.

Extracellular

NH$_2$

Intracellular

COOH
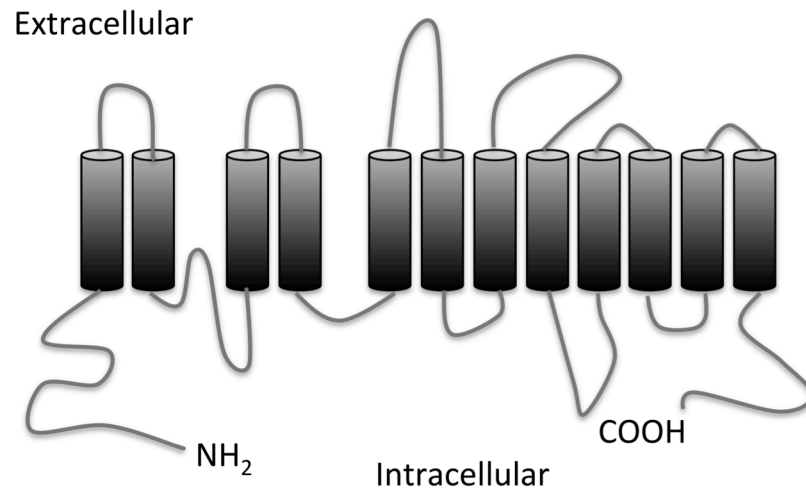
Figure 2 Regulation of glutamate and cystine through action of system xc-
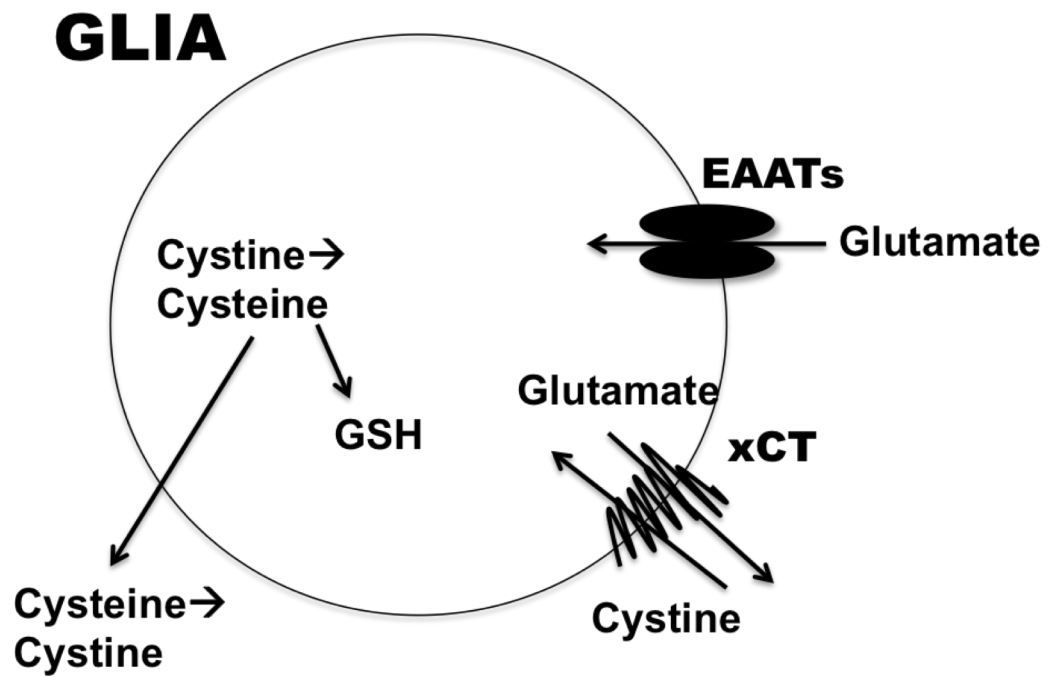
1.2 *In-vitro* studies on system xc-

The first studies on system xc- conducted in cell cultures of human diploid fibroblasts (Bannai and Kitamura, 1980; Bannai, 1986; Murphy et al., 1989). Bannai et al (Bannai and Kitamura, 1980) showed that there was a cystine transporter system that was dependent on extracellular glutamate levels. It was also shown that the reverse was true of this transporter, in low levels of cystine there was no extrusion of glutamate (Bannai and Kitamura, 1980; Bannai, 1986; Murphy et al., 1989). Another one of the initial studies characterizing system xc- noted that sodium-independent "leakage" of glutamate from cells treated with a glutamate uptake blocker and tetrodotoxin (TTX) was most likely due to activity of system xc- (Bradford et al., 1987). System xc- was then studied mostly for its role in cystine intake. Cystine is imported into cells through system xc- and rapidly converted to cysteine which is the rate limiting step GSH synthesis (Murphy et al., 1989; 1990). GSH is an essential antioxidant preventing damage to the cell by reactive oxygen species or peroxides.

Initially it was shown that system xc- was most active in glial cells. Co-culture of glia and neurons showed uptake of cystine in glia and thus GSH synthesis in glia. When neurons were cultured alone their intracellular GSH decreased because unlike glia they did not have a mechanism to internalize cystine (Sagara et al., 1993). It has now been shown that system xc- is expressed in most cell culture lines and helps maintain the synthesis of GSH (Sato et al., 1999). xCT can also be expressed from mouse macrophage cells or

human xCT cDNA in *Xenopus laevis* oocytes. Expression of xCT and 4f2hc in oocytes shows glutamate dependent cystine uptake (Ishii et al., 1991; Sato et al., 2000; Bassi et al., 2001).

Cysteine is a rate-limiting precursor to GSH synthesis, however it is hardly detectable extracellularly because it is oxidized rapidly to cystine in normoxic conditions (Bannai, 1986). Cystine is also important in cystine-cysteine cycling in the cell. Cystine taken up through system xc- is reduced to cysteine, which is used for GSH synthesis or exported back out of the cell through neutral amino acid carriers. Once extracellular, cysteine is quickly converted back to cystine (Bannai, 1984a). Cysteine can only be detected in cell media when there is functional system xc- because it is only extruded when cystine is imported into the cell (Bannai and Ishii, 1982; Bannai, 1984a; Banjac et al., 2007). It has also been shown that the amount of extracellular cysteine-cystine is not affected by the amount of internal GSH, however a reduction in extracellular cystine or cysteine reduces the amount of internal GSH (Anderson et al., 2007).

In-vitro work has shown that when glutamate uptake through EAATs is blocked there are toxic increases in extracellular glutamate due to a reduction in uptake of cystine and intracellular GSH synthesis. Therefore it is thought that system xc- maintains the cellular redox state by both uptake of cystine but also maintaining the amount of glutamate located outside the cell in concert with the action of EAATs taking up excess extracellular glutamate (Figure 2). Prolonged exposure to glutamate in culture leads to cell death through reduced cystine transport (and thus a reduction in GSH) via system xc- (Murphy et al., 1990;

Mawatari et al., 1996). If system xc- or EAATs are disrupted there can be
potentially dangerous increases in glutamate levels which have been implicated
in many types of cell death such as in Parkinson's disease (Lewerenz et al.,
2006; Albrecht et al., 2010).

1.3 Mutants for system xc-

In *Drosophila*, there is an xCT homolog called *genderblind* named for the
bisexual behavioral exhibited by males lacking this gene (Augustin et al., 2007;
Grosjean et al., 2007). The *genderblind* gene shares a 43% amino acid identity
to human xCT (Augustin et al., 2007; Grosjean et al., 2007). Flies lacking the
genderblind gene exhibit reduced glutamate levels as well as an increase in
ionotropic glutamate receptor clustering at the synapse. This shows that in flies
the extruded glutamate from this transporter has important behavioral and
physiological effects (Augustin et al., 2007; Grosjean et al., 2007).

In mice, xCT is encoded by the gene *slc7a11*. This gene is located on the
3rd chromosome and consists of 12 exons. There are currently two mutations in
this gene that are considered null mutants (Chintala et al., 2005; Sato et al.,
2005). The subtle gray mutation (hereafter called *sut*) was first characterized as
a loss of pheomelanin pigment in the C3H/HeSnJ background (hereafter called
SnJ), but was then discovered to be a spontaneous mutation in the *slc7a11*
gene.

Pheomelanin is composed of L-dopa and L-cysteine and produces a red-
brown pigment (Deibel and Chedekel, 1984). Reduction of cystine import

through system xc- causes a reduction in intracellular cysteine and a decrease in pheomelanin production in mouse strains that have a lot of pheomelanin coloration such as C3H/HeSnJ. Injection of sheep *slc7a11* testicularly has also shown an increase in production of brown/yellow patches due to an increase in pheomelanin production through this gene in sheep (He et al., 2012). This mutation in mice resulted in a deletion of the twelfth exon of the *slc7a11* gene followed by an addition of an alternative exon 12 (Figure 3, Adapted from (Chintala et al., 2005; Conrad and Sato, 2012)). According to a Northern blot analysis by Chintala et al., 2005, *sut* mice do not produce *slc7a11* mRNA in the brain due to this mutation, thus it is considered a null mutant for the *slc7a11* gene. However, 3'RACE (Rapid Amplification of cDNA Ends) indicates that there is a possible truncated protein product that could be produced (Chintala et al., 2005).

 sut mice also show a reduction in GSH in melanocytes indicating that loss of cystine intake through system xc- leads to reductions in intracellular GSH in this mutant (Chintala et al., 2005). In culture, astrocytes and meningeal cells derived from *sut* mutants do not proliferate well but can be rescued with β-mercaptoethanol (Shih et al., 2006). It has also been shown that *sut* mice show signs of brain atrophy in adulthood with enlargement of ventricles, thinning of the cortex and shrinkage of the striatum (Shih et al., 2006).

 The Sato lab generated the second mutation in the *slc7a11* gene in 2005 (hereafter referred to as *xCT* knockout mice) (Sato et al., 2005). They inserted a neo cassette and GFP into the 1st exon in a C57BL/6 background (hereafter

called *B6*) and then removed the neo cassette to create an xCT knockout null mutation. Northern blot analysis also confirmed that no mRNA for the gene is produced in these knockouts (Sato et al., 2005) (Figure 4).

xCT mice appear to be fertile and healthy though they have higher plasma levels of cystine than their controls as well as reduced plasma GSH (Sato et al., 2005). Embryonic fibroblast cells from *xCT* knockouts were not able to survive without 2-mercaptoethanol or *N*-acetyl cysteine in the culture media (Sato et al., 2005). *xCT* mice have been shown to have reduced levels of cysteine in cultured neutrophils and rapidly reducing GSH levels over the culture period showing that system xc- plays an important role in neutrophils (Sakakura et al., 2007). *xCT* mice do not have a pigment change similar to *sut* mice because they do not have a coat that is composed of pheomelanin.

 In-vivo, xCT knockout mice do not have lower hippocampal or striatal GSH, increased oxidative stress or brain atrophy as was seen in *sut* mice (Massie et al., 2011; De Bundel et al., 2011). However, *xCT* knockout mice do have a significant reduction in extracellular glutamate levels in the hippocampus and striatum (De Bundel et al., 2011; Massie et al., 2011). They also have an increased limbic seizure susceptibility as well as protection from 6-hydroxydopamine induced neurodegeneration perhaps due to the reduced glutamate levels (De Bundel et al., 2011; Massie et al., 2011).

Human *slc7a11* encodes a protein that is 501 amino acids long and shares 89% sequence identity with mouse xCT (Kim et al., 2001). Human xCT also has a splice variant that encodes a 495 amino acid protein which is highly

expressed in U87 glioma cells but not another glioma cell line SNB-19 (Kim et al.,

2001; Patel et al., 2004)
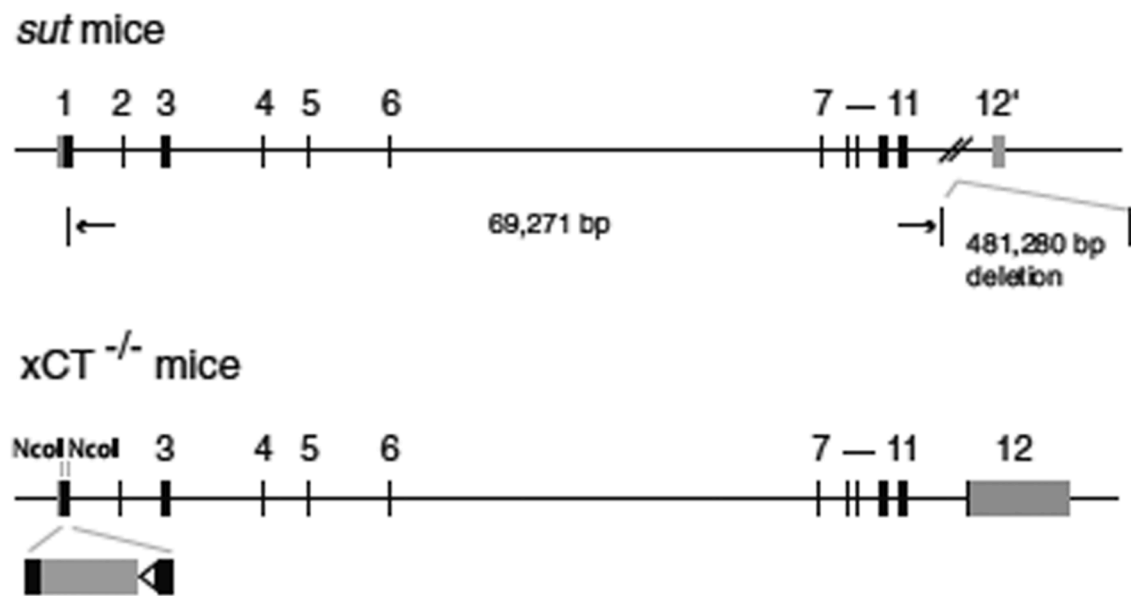
Figure 3 Location of the mutation in *sut* and *xCT* mice (Adapted from Conrad and Sato 2012).

Slc7a11 is 12 exons long. The sut mutation has a deletion in the 12th exon with an alternate 12th exon as depicted. The xCT mutation has a GFP inserted into the 1st exon with a stop codon.

1.4 Transcriptional regulation of slc7a11

The 5' flanking region of *slc7a11* contains four antioxidant response elements (Lewerenz et al., 2009), one of which corresponds to the transcription factor Nrf2 (Sato et al., 2004). The other three antioxidant response elements appear to be related to oxidative stress, including oxygen and diethyl maleate (Bannai, 1984b; Bannai et al., 1989; Hosoya, 2002; Sasaki, 2002; Sims et al., 2012). Upregulation of Nrf2 by *tert*-butylhydroquinone (*t*-BHQ) strongly increases xCT protein levels and activity of system xc- in the hippocampal cell line HT22 (Lewerenz et al., 2012a). Retroviral overexpression of Nrf2 in rat astrocyte cultures also showed a large increase in xCT protein further demonstrating that increases in Nrf2 expression increases xCT protein and activity (Shih et al., 2006).

In addition to the antioxidant response elements, a pair of amino acid response elements (AAREs) has been found in the *slc7a11* gene. Guanosine also seems to increase expression of *slc7a11*. When guanosine was added to HT22 cells, there was an increase in both system xc- activity and GSH in the presence of glutamate (Albrecht et al., 2013). One of the AAREs has been identified to be ATF4, which upregulates the transcription of xCT mRNA, protein levels and system xc- activity (Lewerenz and Maher, 2009). ATF4 has been shown to be regulated by phosphorylation of the translation initiation factor eIF2α (Lewerenz et al., 2009; Lewerenz and Maher, 2009). Phosphorylation of eIF2α is regulated by amino acid deprivation, therefore through action of eIF2α on ATF4,

system xc- can be upregulated to deal with amino acid deprivation (Sato et al., 2004; Lewerenz and Maher, 2009; Lewerenz et al., 2012b).

1.5 Location of xCT/*slc7a11* expression in the mouse brain

Sato et al (Sato et al., 2002) showed via *in situ* hybridization that xCT is highly expressed in the area postrema, subfornical organ, habenular nucleus, hypothalamic area, ependymal cells of the 3rd lateral ventricle, and meninges of the adult mouse brain. This is consistent with the Allen Mouse Brain Atlas Project *in situ* hybridization data (Figure 3). The Allen Mouse Brain Atlas also shows increased expression in the cerebellum and olfactory system as can be seen in Figure 3. xCT has also been shown to be expressed in other parts of the body such as the pancreas, intestines, pituitary, retina, and most cell lines (Ishii et al., 1991; Bassi et al., 2001; Bridges et al., 2001; Pow, 2001). Western blot analysis shows that xCT is highly expressed in the meninges but also in the cortex, hippocampus, ventricle, striatum, and cerebellum (Shih et al., 2006).

There is some contention about whether xCT is expressed solely on glia or also on neurons, Burdo et al (Burdo et al., 2006) showed expression of xCT in neurons and glia in the brain while Pow only detected presence of xCT in glial cells and not neurons (Pow, 2001). However, the study by Pow only used retinal cells as a control and not brain slices as well as a selectively transported substrate aminoadipic acid, so it is unclear if this method is also specific to other CNS tissue besides retina (Pow, 2001). Whether on neurons or glia, system xc- is important for helping maintain extracellular glutamate concentrations and synaptic function in neurons and glia.
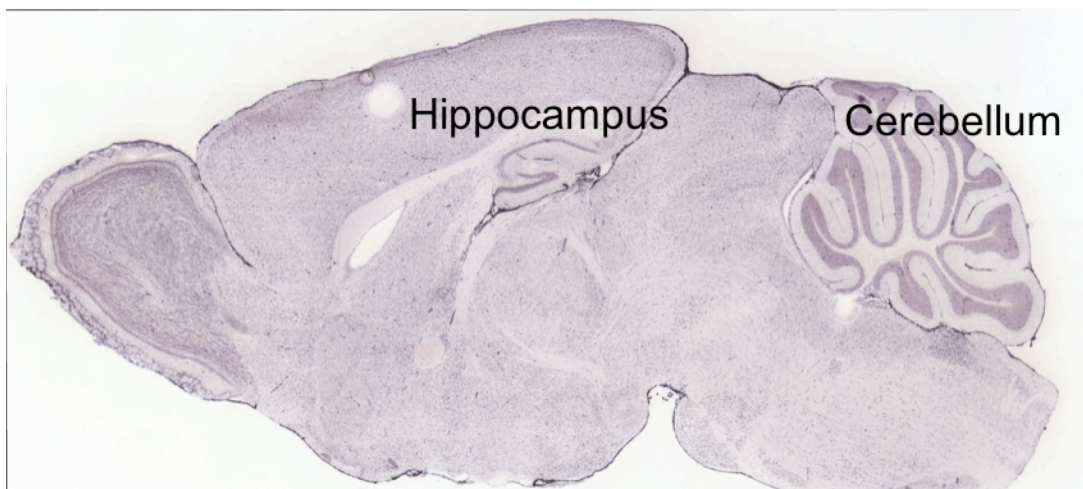
Figure 4 Allen Brain Atlas *in situ* hybridization data depicting the expression of *slc7a11* mRNA in a sagittal section of mouse brain.

Note there is increased expression in the cerebellum and hippocampus of the mouse brain as well as meninges (http://mouse.brainmap.org/gene/show/26317).

1.6 Sources of extracellular glutamate

There are two main pools of glutamate in cells, synaptic glutamate and extrasynaptic glutamate. Glutamate is widely used in the nervous system as a neurotransmitter, more than 80% of synapses use glutamate as the primary neurotransmitter (Diamond and Jahr, 2000). Synaptic glutamate accumulates in vesicles at nerve terminals and is released into the synaptic cleft after the arrival of action potentials. This released glutamate then activates receptors on the post-synaptic cell. Synaptic glutamate is confined to the synaptic space by astrocytes, which quickly take up excess glutamate from the cleft through the activity of excitatory amino acid transporters (EAATs) (Diamond and Jahr, 2000). Synaptic glutamate taken up by glia cells is then converted to several bi-products including glutamine, which is then released to be taken up by neurons and synthesized back into glutamate (Marcaggi and Attwell, 2004; Allaman et al., 2011; Rodriguez et al., 2013).

It was originally thought that extrasynaptic glutamate concentrations originated from synaptic glutamate that diffused away from the synapse (Asztely et al., 1997). However it has been shown that there are other non-neuronal sources of glutamate that can account for the amount of glutamate maintained extrasynaptically, such as glutamate release from system xc- (Baker et al., 2002; Augustin et al., 2007; Grosjean et al., 2007; Massie et al., 2011; De Bundel et al., 2011).

1.7 xCT as a regulator of extracellular glutamate

There have been several studies that have shown upon inhibition of system xc-, either through mutations or pharmacologically, there are drastic reductions in extracellular glutamate levels (Baker et al., 2002; Augustin et al., 2007; De Bundel et al., 2011; Massie et al., 2011). Baker et al used a system xc- inhibitor S-(4)-CPG in the rat striatum and showed a 60% reduction in extracellular glutamate levels detected through microdialysis (Baker et al., 2002). Dr. Ann Massie's group has shown reductions in extracellular glutamate in xCT knockout mice in both the striatum and hippocampus compared to their wildtype littermates using microdialysis (De Bundel et al., 2011; Massie et al., 2011). Studies in *Drosophila* have shown that elimination of system xc- shows a 50% reduction in extracellular glutamate levels and may have an important role in modulating synaptic activity (Augustin et al., 2007).

Dr. Cavelier proposes that in normal conditions, system xc-'s release of glutamate would be fairly low due to low physiological levels of cystine (Cavelier and Attwell, 2005; Cavelier et al., 2005). Most studies on system xc- add cystine during their measurements of glutamate or use methods such as microdialysis which may introduce cystine from the blood through disruption with the cannula (Cavelier et al., 2005; Cavelier and Attwell, 2005). There are of course several other sources of extracellular glutamate, as well as system xc-, such as EAATs, swelling-activated anion channels, calcium-dependent astrocytic release, P2X$_7$ receptors, hemichannels, and already discussed, synaptic glutamate (Rodriguez et al., 2013).

1.8 Other regulators of extracellular glutamate levels

There are several other sources of extracellular glutamate that could be important regulators of the overall levels of non-synaptic glutamate besides system xc-. The two primary sources discussed here are excitatory amino acid transporters (EAATs) and astrocytic glutamate release. These two systems are sodium-dependent (EAATs) and calcium-dependent (glial release), and therefore extracellular glutamate that is sodium and calcium independent cannot be attributable to these mechanisms and has been attributed to system xc- (Baker et al., 2002; Featherstone and Shippy, 2007).

There has been a lot of evidence recently that glia release vesicular glutamate in a fashion similar to neuronal transmission (Bezzi et al., 1998; 2004; Haydon et al., 2009). An increase in intracellular calcium concentration is sufficient to cause glutamate release from astrocytes (Parpura et al., 1994). These calcium transients seen in astrocytes originate from activation of metabotropic glutamate receptors following neurotransmitter release from neurons (Parpura et al., 1994). Additional evidence for calcium-dependent astrocytic glutamate release is from caged calcium experiments showing that physiological levels of calcium can cause astrocytic glutamate release as well as evidence from acute slice preparations (Bezzi et al., 1998; Innocenti et al., 2000; Bezzi et al., 2004).

It is thought that astrocytic release may be important for synaptic regulation and coordination of neuronal activity and also important in maintaining

extracellular glutamate levels (Rodriguez et al., 2013). A limited number of experiments have determined the amount of extracellular glutamate contributed by astrocytic glutamate release and to show exocytosis of glutamate via astrocytes *in-vivo* (Hamilton and Attwell, 2010). In addition to astrocytic release of glutamate, EAATs are thought to regulate glutamate levels through uptake of glutamate extracellularly or reversal of uptake.

The primary mechanism through which EAATs are thought to play a role in extracellular glutamate levels is through reversal of uptake of glutamate (Rodriguez et al., 2013). EAATs traditionally are responsible for removal of glutamate following release through neurotransmission or action of system xc- (Jabaudon et al., 1999). EAATs are sodium-dependent transporters, which couple transport of sodium and potassium down their concentration gradients to drive glutamate into the cell (Suchak et al., 2003; Allaman et al., 2011). Astrocytes exhibit primarily two types of EAATs that take up glutamate, EAAT-1 or GLAST and EAAT-2 or GLT-1 (Danbolt, 2001). It is thought that in conditions of ischemia where potassium levels outside the cell are abnormally high, EAATs can act in reverse, pumping out glutamate into the extracellular space (Malarkey and Parpura, 2008). The resulting higher level of extracellular glutamate can lead to glutamate-induced toxicity (Malarkey and Parpura, 2008). In mice that are lacking EAAT-2, prolonged exposure to ischemia did not lead to neuronal death as compared to their controls (Mitani and Tanaka, 2003). This showed that as predicted this EAAT can act in reverse when under prolonged ischemic conditions and contribute to the ischemic event (Mitani and Tanaka, 2003).

However, under short-term acute ischemia EAAT-2 knockouts tended to have higher extracellular glutamate levels due to loss of uptake of glutamate (Mitani and Tanaka, 2003).

Another potential mechanism by which EAATs can influence ambient levels of glutamate is through regulation of transporter number on astrocytes. Reducing the number of EAATs is expected to reduce glutamate uptake and therefore increase the levels of extracellular glutamate as contributed by system xc- and synaptic release (Rothstein et al., 1996). The loss of EAAT-1 or EAAT-2 through the use of antisense oligonucleotides to knockdown protein expression produced elevated extracellular glutamate levels in the striatum (Rothstein et al., 1996). ENT-1 (the equilabrative nucleotide transporter) knockout mice have increased extracellular glutamate potentially due to a reduction in expression of EAAT-2 (Lee et al., 2012). However, it has been shown through Western blotting that in xCT mutants, there is not a decrease in the number of EAATs (De Bundel et al., 2011; Massie et al., 2011). Glutamate levels in xCT knockout mice tend to be half that of wildtype littermates perhaps due to normal function of EAATs and no system xc- to export glutamate through (De Bundel et al., 2011; Massie et al., 2011). However, the relative functioning of EAATs, and not just abundance of the transporter in system xc- mice has not been studied.

In addition to being important in maintaining extracellular glutamate levels, EAATs also transport cysteine and could have some functional importance in the redox balance that system xc- also participates in (Zerangue and Kavanaugh, 1996; Flynn and McBean, 2000; McBean, 2002; Chen and Swanson, 2003;

Hayes et al., 2005). EAAT-3 or EAAC-1, which is located on neurons, is thought

to be more important in cystine uptake than in glutamate reuptake (Valdovinos-

Flores and Gonsebatt, 2012).

1.9 Changes in synaptic function related to reduced extracellular glutamate

There was a lot of debate regarding the role (if any) of extrasynaptic

glutamate in synaptic function and plasticity (Parpura et al., 1994; Haydon et al.,

2009). Now it seems fairly clear that extrasynaptic glutamate is an important

regulator of both ionotropic receptors, NMDA and AMPA, and in G protein-

coupled receptors (Moran, 2005; Augustin et al., 2007; Grosjean et al., 2007;

Haydon et al., 2009; Li et al., 2012b). Astrocytes are closely located to neurons,

so it is not that surprising that glutamate released through system xc- might have

effects on nearby pre and post-synaptic cells (Eroglu and Barres, 2010).

1.10 Actions of ambient glutamate levels on ionotropic receptors

Originally the action of EAATs on synapses was studied through the

glutamate uptake inhibitor threo-beta-Benzyloxyaspartate (TBOA). TBOA

application tended to increase slow transient currents that are mediated by

NMDA receptors (Angulo et al., 2004).   It was also found that activation of

pyramidal neurons by glia tended to synchronize activity of other neurons nearby

(Angulo et al., 2004). Blockage of EAATs by TBOA has also shown an increase

in extracellular glutamate levels (Jabaudon et al., 1999). This increase is sodium

and calcium independent meaning that it is likely attributable to system xc-

(Jabaudon et al., 1999). Does glutamate released from system xc- have effects on synaptic function?

In *Drosophila*, mutation of the homologous gene for murine system xc-, *genderblind* (*gb*), leads to a 50% reduction in extracellular glutamate levels as well as a doubling of functional synaptic glutamate receptors (Augustin et al., 2007). In this study, the GluR2A and GluR2B subunits of AMPA receptors were increased in response to lower ambient extracellular glutamate levels. This rise in glutamate receptor number was rescued by culture of *gb* synapses in wildtype glutamate concentrations. Beyond just immunocytochemistry, spontaneous excitatory junction current (sEJCs) amplitude was also increased in *gb* mutants showing that functionally as well as structurally *gb* mutants differ from controls (Augustin et al., 2007).   The implications of this study are that under normal levels of extracellular glutamate, some of the glutamate receptors are constitutively desensitized which suppresses their ability to cluster at the synapse. When glutamate levels are abnormally low, as in *genderblind* mutants, there is a rise in glutamate receptor number as well as sEJC amplitude.

A study by Warr et al in rat brain slices showed that cystine application on Purkinje cell preparations invoked a current that is probably related to non-NMDA glutamate receptors due to system xc- increases in extracellular glutamate (Warr et al., 1999). This inward current was abolished by application of CNQX an inhibitor of non-NMDA receptors. This current was unaffected however by TTX or zero calcium suggesting that this current is sodium and calcium independent and not evoked by nearby cells. However, they failed to block the current

through inhibition of system xc- through 1mM DIDS (4,4-diisothiocyanatostilbene-2,2'-disulfonic acid) and furosemide that have been shown to be system xc-inhibitors but also have non-specific effects (Warr et al., 1999; Cavelier and Attwell, 2005). However, all of this data was collected on slice preparations from rats and not on xCT knockout mice.

There has been one study to date that has looked at synaptic transmission in a system xc- knockout. Li et al used *sut* mutant mice to measure synaptic changes related to system xc- in a knockout mouse model (Li et al., 2012b). They showed that long-term potentiation (LTP) is reduced in the hippocampus of *sut* mutants compared to their wildtype controls (*SnJ*) using extracellular field potential recordings. Basal synaptic transmission was not impaired in *sut* mice nor did application of extracellular glutamate rescue the reduction in LTP. They also could not show a reduction in LTP in wildtype mice treated with S-CPG, a system xc- inhibitor. Paired-pulse facilitation which is mediated by pre-synaptic mechanisms is also not impaired in *sut* mice (Li et al., 2012b).   The other major synaptic effects that system xc- is thought to regulate is tone of G protein-coupled metabotropic receptors.

1.11 Tonic activation of metabotropic receptors

System xc- is also potentially a regulator of synaptic function through tonic activation of G protein-coupled metabotropic glutamate receptors (mGluRs). In *gb* flies as discussed above, it was shown that *gb* causes an increase in AMPA receptor number and activation and metabotropic receptors activation as well

(Grosjean et al., 2007). There are two main types of mGluRs, group I (mGluR1/5)

and group II (mGluR2/3) in mammals. Group I mGluRs are typically located

presynaptically and are associated with activating phospholipase C. Group II are

located postsynaptically and are inhibitors of adenyl cyclase (Kalivas, 2009). It

was originally thought that mGluRs would be regulated by extracellular glutamate

because they are typically located towards the outside of the synapse. It is

thought that synaptically located receptors like AMPA and NMDA may be less

accessible to extracellular glutamate due to the size of the synapse (Kalivas,

2009).

An *in vitro* study by Moran et al showed that activation of system xc- in the

nucleus accumbens core, by adding cystine, stimulates group II mGluRs

(mGluR2/3) (Moran, 2005). Activating mGluR2/3 caused a reduction in the

synaptic release probability (Moran, 2005). Thus the amount of glutamate

released by system xc- can directly impact future release events on nearby

neurons through tonic activation of group II mGluRs. The majority of the studies

involving the regulation of mGluRs by system xc- are related to the importance of

glutamate homeostasis in addiction paradigms (Kalivas et al., 2003; Kalivas,

2009). These were some of the first studies looking at how extracellular

glutamate affects neuronal transmission and ultimately behavior. What evidence

is there that system xc- regulates behavior?

1.12 Behavioral changes related to loss of extracellular glutamate through xCT

There have been several studies in different model systems that have shown that system xc- has affects on behavior. However, there is not a specific behavioral deficit in mice that can be directly attributed to system xc- as discussed later. So what behaviors to date have been attributed to system xc-?

1.13 *Drosophila* Behavior

In *Drosophila*, a consequence of the *genderblind* (*gb*) mutation and the reason for the interesting name, is that male flies that were missing system xc- were bisexual. Male fruit flies have a very distinct and ritualized courtship. They first tap the female to evaluate pheromones via chemoreceptors on his leg, then sing a species-specific courtship song, and lick her genitalia to sample pheromones. If she deems him acceptable they will then mate. Interestingly, *gb* mutants extended this courtship behavior indiscriminately to both male and female flies (Grosjean et al., 2007).

This bisexual behavior in flies was attributed to the misinterpretation of chemical cues caused by changes in glutamate signaling through system xc-. To further support the role of system xc- in this behavior they showed that this behavior could be induced by conditional RNAi to the *gb* gene in just a few hours. This indicates that synaptic strength (an increase in ionotropic glutamate receptors number) can be modified on a few hour timescale in flies through deactivation of this transporter (Grosjean et al., 2007).

Consistent with system xc- affecting behavior, when *gb* flies were given an ionotropic glutamate receptor antagonist, thus reducing synaptic strength, their homosexual courtship behavior decreased. As stated before, this increase in synaptic strength is most likely due to decreased desensitization of AMPA receptors leading to higher sustained expression. When wildtype flies were given an AMPA receptor desensitization inhibitor, concanavalin A, which would increase AMPA receptors similar to *gb*, they also exhibited increased homosexual courtship. It was also shown that a metabotropic receptor antagonist decreased homosexual behavior in *gb* flies indicating that it is through both ionotropic and metabotropic mechanisms that his behavior is mediated (Grosjean et al., 2007). Flies are a great model system since their behavior and synaptic strength can be easily and quickly manipulated through genetic or pharmacological manipulations. There have been several studies on system xc- and it's effects on mammalian behavior, however many of these studies used drug manipulations only or looked at one genotype of system xc- mutant.

1.14 Mouse Behavior

The original studies of behavior attributable to system xc- were focused on the relationship of glutamate homeostasis and drug seeking behaviors in rats (Cornish and Kalivas, 2000; Baker et al., 2002; Kalivas et al., 2003). Repetitive cocaine administration shows a reduction in basal levels of glutamate in the nucleus accumbens and an increase in reinstatement behaviors when animals are introduced to cocaine again. This could be due to down-regulation of system

xc- and it's effects on mGluRs (Cornish and Kalivas, 2000). Drs. Baker and

Kalivas have shown that when system xc- is down-regulated after cocaine abuse

in rats, by applying a system xc- agonist (*N*-acetyl cysteine (NAC)), animals no

longer perform reinstatement behaviors when reintroduced to cocaine (Baker et

al., 2003b) (Figure 5). This shows that system xc- may be important in cocaine

reinstatement and is downregulated during cocaine abuse. Cocaine-dependent

humans also given NAC showed a reduction in craving for cocaine after an IV-

administered injection of cocaine (Amen et al., 2011). This cocaine

reinstatement reduction behavior can be blocked in rats when they are

administered both NAC and (*S*)-4-carboxyphenylglycine (CPG) a system xc-

inhibitor and given access to cocaine (Kau et al., 2008). Similarly, blocking

mGluR 2/3 prevented the action of NAC on the reinstatement behavior of rats

addicted to cocaine (Moran, 2005).

Similar results were also found in relationship to nicotine administration.

Repetitive administration of nicotine leads to a reduction in the xCT protein in the

nucleus accumbens. Also human smokers treated with NAC reported a

reduction in the number of cigarettes smoked (Knackstedt et al., 2009).

There has been some behavioral analysis of system xc- and knockout

mice, however nothing that was tested in both strains of knockout (*sut* and *xCT*).

To complement their electrophysiology data, Li et al performed fear conditioning

and passive avoidance tasks on *sut* mice (Li et al., 2012b). They showed that

sut mice have impaired cued and contextual fear conditioning compared to

wildtype (*SnJ*) as well as impaired learning of fear-related contextual cues (Li et

al., 2012a).   However, this study only measured these behavioral tests in *sut*

mice, and only in male mice.

Dr. Ann Massie's group has shown behavioral effects of system xc- in *xCT*

mice compared to their background *B6* (De Bundel et al., 2011). They tested

animals in a Morris Water Maze, open field maze, 3-arm spontaneous alternation

task, and a delayed alternation task. They showed no impairment of *xCT* mice in

the open field maze or Morris water maze. However, they did show that young

xCT knockout mice made fewer alternations in the 3-arm alternation task and

had fewer arm choices in the novel arm during the delayed spontaneous

alternation task. This could potentially indicate impairment in spatial working

memory, however the old *xCT* mice did not show this deficit in spatial memory

and there were no impairments in the Morris water maze.   This group also only

used male mice and no other genetic backgrounds for the mutation such as *sut*

mice (De Bundel et al., 2011).   Many of these studies looking at behavior in

system xc- mutants or other organisms use pharmacological means to

manipulate system xc-. What are some of the ligands and inhibitors to system

xc- and how do they work?

Figure 5, Diagram showing how NAC is used in cocaine addiction.

After a rat becomes addicted to a drug, they show decreases in system xc-activity, decreased basal glutamate levels and reduced tone of mGluR 2/3. When given NAC prior to reinstatement of drug, the animals no longer attempt to get the drug suggesting that NAC ameliorates the effects of a reduction in system xc- activity.

NAC delivery

Rat becomes addicted to drug → **1. Decrease in system xc-**
2. Decrease glutamate
3. Decrease tone of mGluR2/3 → **Rat does not press lever for cocaine**

Reintroduce drug
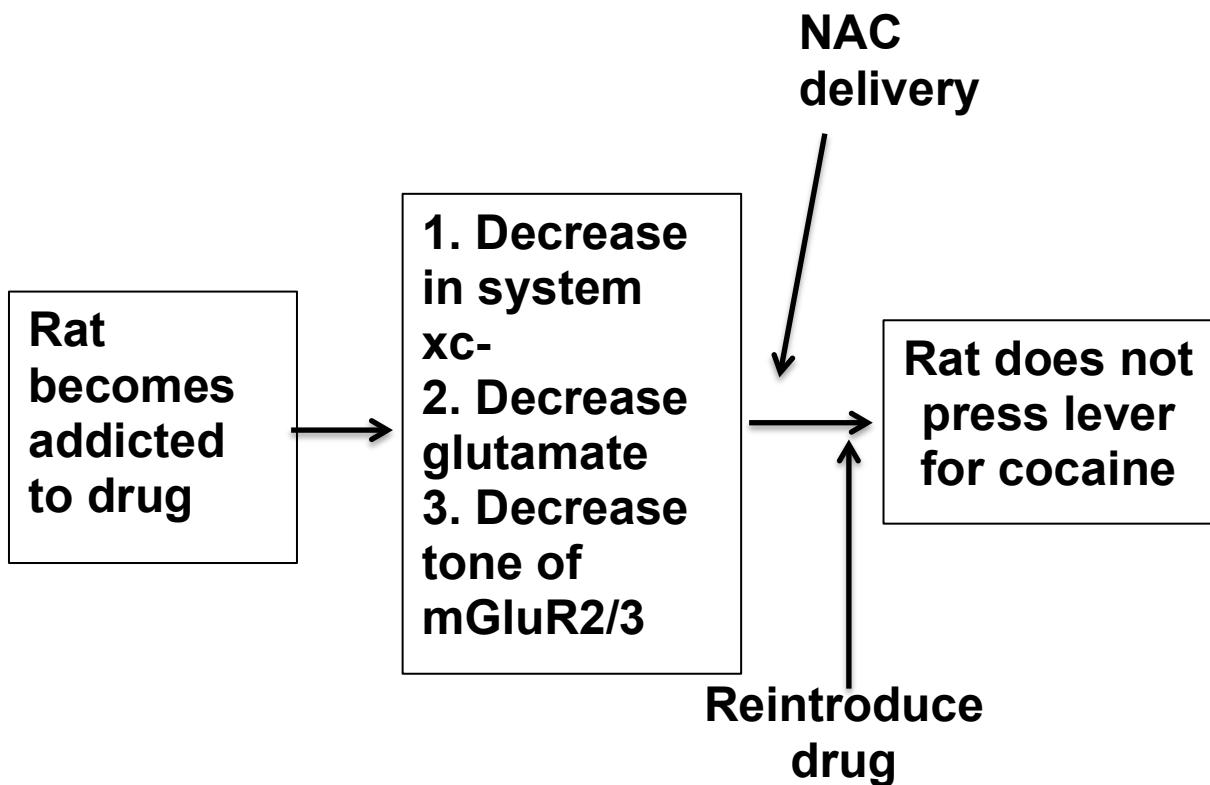
1.15 Pharmacological manipulation of system xc-

As discussed previously, the main regulators of system xc- function are glutamate and cystine concentrations, however there are other molecules that bind to system xc- and change it's function and regulation. N-acetylcysteine (NAC) has been shown to be one of the primary ligands for system xc- activity. NAC is an antioxidant that is used in treating acetaminophen overdoses, preventing environmental pollutants, and many other diseases, mostly related to its antioxidant properties (Baker et al., 2003b). Ceftriaxone, β-lactam antibiotic, is also thought to be a transcriptional regulator of system xc-, with a possible role in mediating a normalization of glutamate levels following cocaine addiction (Lewerenz et al., 2009; Trantham-Davidson et al., 2012).

There are several inhibitors that researchers have used to block system xc-. Among the most potent are (S)-4-carboxyphenylglycine (S-CPG) and sulfasalazine (SAS) (Ye et al., 1999; Patel et al., 2004). S-CPG was initially characterized as a group I metabotropic receptor antagonist further demonstrating the non-selectivity of these inhibitors (Bedingfield et al., 1995). SAS and S-CPG were first demonstrated as inhibitors for system xc- by their role in glial tumors (Ye et al., 1999; Chung et al., 2005).   SAS was initially used as an anti-inflammatory agent in Chron's disease and inflammatory bowel disorders (Chung et al., 2005). Other inhibitors of system xc- include an AMPA receptor agonist, (RS)-4-Br-homoibotenate, a kainite receptor agonist, (RS)-5-Br-willardiine, and ibotenate, a NMDA and metabotropic receptor agonist. However, these other inhibitors do not seem to be as potent as either SAS or S-CPG and

all are agonists of glutamate receptors and have non-specific effects (Bridges

and Patel, 2009).

1.16 Summary and aims of present study

To date there has not been a complete behavioral characterization of

system xc-. System xc- knockouts should have potent effects on behavior either

through cell death due to loss of neuroprotection, reduced tone of metabotropic

glutamate receptors or increased postsynaptic ionotropic glutamate receptors.

Many of the current studies have looked in only one knockout strain of system

xc- or used pharmacological manipulations to measure behavior. I will determine

the effects of loss of system xc- through behavioral characterization of both *sut*

and *xCT* mice and a cross of the two, *xCT/sut* mice.

2 METHODS

2.1 Molecular Techniques

2.1.1 Mouse tail extraction:

DNA was extracted from mouse-tails. Briefly, 0.5cm of tail was placed into a microcentrifuge tube, and 500µL of digestion buffer is added, vortexed and incubated overnight at 55°C. Digestion buffer consists of 50mM Tris-HCl (pH 8.0), 100mM EDTA (pH 8.0), 0.5% SDS and 500µg/mL Proteinase K. Next 500µL phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by inverting the tube at least 20 times. The mixture was then spun in a microcentrifuge at top speed for three minutes, and using a P-1000 pipet tip with the tip cut off, the upper aqueous layer/phase was transferred to a new microfuge tube (along with the viscous layer). Next the mixture was extracted with chloroform/isoamyl alcohol (24:1) as above, the aqueous layer and interface should no longer be viscous, if it was, it was left behind. After centrifuging, 50µL of 7.5M NH_4OAc and 1mL 100% ethanol (non-denatured) were added and inverted 10-20 times to precipitate high molecular weight DNA. DNA precipitated as a white or brown clump. A pipet tip was used to gently transfer the DNA to a new tube containing 500µL 70% ethanol and mixed by inverting a few times and spun at top speed for ten minutes.   Most of the ethanol was then aspirated off then quickly spun. Afterwards any residual ethanol was removed with a pipet tip and left to air dry for ten minutes before 200µL of H_2O was added to the pellet.

The DNA was then incubated for one hour at 50°C then gently pipetted to resuspend any undissolved DNA. It was then stored at -20°C until used in genotyping.

2.1.2 PCR

DNA was extracted as described above. Primers localized for the 12th exon of the *slc7a11* gene were generated to detect *sut* mutants, a control primer was also generated just downstream of the *slc7a11* gene on the 3rd chromosome; both are listed below, forward primer followed by reverse.

sut- GCTGCTCATGGTAGGCTTTC TGCATCTCCATCCATGTTGT

control- GCCTGACTTCTGGCTGGTAG GTCCAAGAGAGGCTGCAAAC

The following PCR reaction was used. A master mix containing 35.8µL H_2O, 1.5µL 50mM $MgCl_2$, 5.0µL10x Invitrogen –$MgCl_2$, 1.0µL of 10mM of each of the primers, 0.2µL 50mM dNTP, 0.5µL Taq Polymerase, and 3.0µL genomic DNA. The following PCR protocol was followed. The samples were placed into the PTC-200 (MJ Research) initially at 95.0°C for 3 min, followed by the following cycle, 95.0°C for 0.5 min, 58.5°C for 0.5 min and 72.0°C for 2 min repeated 39 times. After the cycle, the samples were maintained at 72°C for 10 min followed by 4°C for 1 min. Once the PCR reaction was complete, samples were mixed with 10µL of loading dye and run on a 2% agarose ethidium bromide gel. The control primers produced a band around 1.0kb, the *sut* primers produced a band around 0.8kb. An example of the PCR products is shown in Figure 1.

Figure 1 PCR results for sut reaction.

Lane 1 shows a ladder that was run with the gel. Lane 2 is an example of a control animal whereas lanes 3, 4, 5 and 6 are sut mutants as depicted by only control bands at 1.0kb.
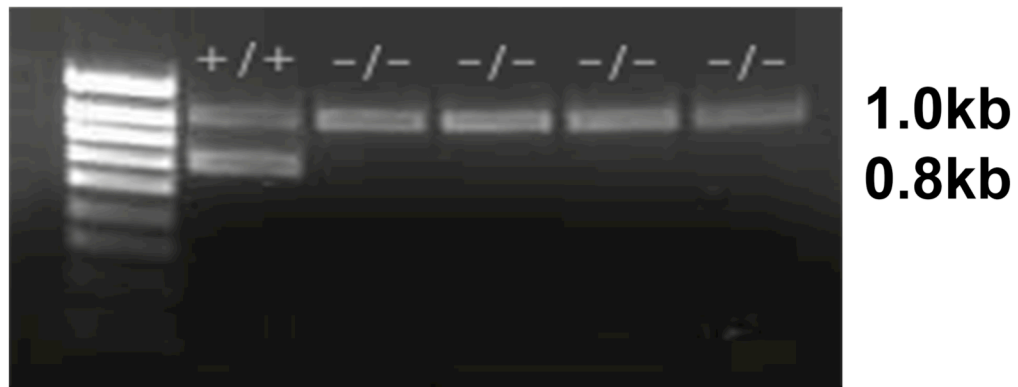
2.1.3 Western blotting

Procedures for blotting and brain homogenization were followed from Massie et al., 2008. Brains of *SnJ, sut, B6, xCT, B6/SnJ,* and *xCT/sut* mice 70-100 days old were extracted and homogenized as described below. Brains were homogenized using a cold mortar and pestle in 1000µL extraction buffer. Extraction buffer contained 2% Sodium dodecyl sulfate, 60mM Tris Base pH 6.8, 100mM dithothreitol, and 1mM Na$_2$EDTA. After homogenization, samples were incubated for 30 minutes at 37°C. Samples were processed four times through 20G needles and ground again with a small pestle in a 1500µL tube. Samples were then spun at 10,000G at 4°C and supernatants were stored at -20°C until use in Western blotting. Samples were then boiled for 5 minutes at 95°C. A Bradford (Biorad) was then done to determine protein concentrations. 10 micrograms of protein and SDS mix were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (4-20% gel; Bio-rad) with a Kaleidoscope pre-stained standard for a ladder (Bio-Rad). The electrophoresis was run at 100mV with 1x running buffer. The gel was then transferred to a polyvinylidene fluoride membrane (Immobolin-P, Millipore) using a Criterion Blotter (Bio-Rad), which was run with 1x transfer buffer at 100mV for one hour. To confirm that the transfer was successful a Coomassie stain was done on the gel, and a Ponceau stain was done on the PVDF membrane. Following protein transfer confirmation, non-specific binding was blocked by incubating the membrane for one hour with 5% Non-fat milk and PBTX. The membrane was incubated overnight at 4°C with 1:1,000 xCT antibody (Novus) or 1:1,000 for

EAAT-1,2,3 (Novus) and a control of 1:2,000 anti-actin (Millipore) in 5% non-fat milk and PBTX. The next day, the membrane was incubated for one hour with horseradish-peroxidase-conjugated anti-rabbit Immunoglobulin (1:50,000; Promega Madison, WI for EAAT 1, 2 or 3 or 1:100,000 for xCT) and anti-mouse immunoglobulin (1:50,000; Promega Madison, WI for actin). The membrane was then visualized with Clarity West ECL substrate (Biorad). Between each incubation step and illumination; the membrane was washed three times for ten minutes with PBTX.   Densities of immunoreactive bands were compared to the actin bands on the same membrane and were repeated at least three times for each genotype and sex.

2.2 Microdialysis surgeries

6-7 males between the ages of 90-120 days old of *sut*, *SnJ*, *xCT*/*sut* and *B6/SnJ* were used for microdialysis experiments. 6-7 90-120 day old female *xCT/sut* and *B6/SnJ* mice were also used. Estrus slides were taken on the same day samples were collected from females. Mice were anaesthetized I.P. according to their weight with a solution containing 1mL ketamine, 0.5mL Rompun 2% (xylazine), and 8.5mL saline. For example, one male mouse weighed 25.0g. It was given 0.23mL of anesthesia solution initially, followed by doses every five minutes of 0.02mL until it seemed to not perceive pain as measured by a foot pinch. For this particular mouse it took two extra doses for a total dose of 0.27mL anesthesia. Once the mouse was anesthetized it was placed onto the stereotaxic frame to ensure proper probe placement.

An incision was made through the middle of the head to expose the skull. Clamps were placed to keep the skin away from the skull. The membrane surrounding the skull was scraped with a scalpel to expose the scalp. Two marks were initially made with marker at the probe coordinates using Bregma as described below (Table 1). A 6 gauge needle was used to create holes in the skull in the areas marked for probe placement. Two screws were also placed contralateral to the probe guides to keep the putty from coming off. Probe guides were placed in the striatum and cerebellum according to the coordinates below relative to Bregma (Table 1). Dental putty was then used to secure the probe guides on the skull and to keep the wounds from being exposed to contaminants.

	Striatum	Cerebellum
Lateral	-2.0	+2.0
Anterior-Posterior	+0.6	-6.1
Vertical	+1.8	+0.7

Table 1, Localization of probe placement for the striatum and cerebellum.

Animals were either placed in cages to recover (if the Rat-turns were already occupied) or probes were introduced right away while animals were still anesthetized and put into the rat-turns to make sure that they did not get tangled in their tubing. If animals were allowed to recover before probe introduction, several hours after surgery, they were restrained and probes were introduced followed by the animal being placed in the rat-turn. Two surgeries were conducted for each experimental group, one control animal and a knockout animal to control for daily environmental differences.

Probe sampling: A 2mm probe was used for glutamate collection in the striatum, while a 1mm probe was used for the cerebellum. Once probes were inserted animals were allowed to recover from anesthesia before sample collection. Once animals were awake, the flow through the microdialysis tubing was slowed to 0.07mL/min and collection was taken overnight to obtain slow-flow samples. The microdialysis tubing contained a 2.3 mM Ca Ringer's solution. The Ringer's solution contained 4.3g NaCl, 0.150g KCl, and 0.200g $CaCl_2$:$6H_2O$ which was then filtered and stored at 4°C, it was used for no longer than one week. The morning following surgery, a second sample at 0.07mL/min was taken for three hours in case the tubing became blocked overnight and the first sample was not recovered. Following slow flow collection, around 10am, the flow was increased to 0.2mL/min and 12 samples were collected every twenty minutes. 15µL were then transferred to a glutamate collection tube and stored in 4°C until frozen at -20°C after collection was complete. Several mice mysteriously died during sample collection, the samples around the time the mice

were acting unusual and prior to their death were excluded from the experiments. Following sample collection mice were given 1mL of pentobarbital until they were no longer breathing and it was determined they were dead. Their brains were then removed and stored in 10% paraformaldehyde at 4°C until probe placement could be confirmed. Probes were sometimes used twice before discarding if they looked okay and sample collection went well.

2.3 HPLC

High Pressure Liquid Chromatography was performed with fluorescence detection to determine glutamate and aspartate levels in samples from mouse microdialysis (as described in (Van Hemelrijck et al., 2005). Liquid chromatography was performed using a Shiseido C_{18} capcell pak MG reverse phase column. Fluorescence detection was performed with a Shimadzu spectrofluorometric detector RF-10Axl. Integration of chromatograms was performed by an integration computer program KromaSystem 2000 (Kontron, Italy). Glutamate and aspartate were measured using the following conditions. Mobile phase A consisted of 0.025M sodium phosphate ($Na_2HPO_4-2H_2O$) buffer pH 9.0 and 1%Tetrahydrofuran (THF). Mobile phase B contained 90% MeOH and 10% water.

2.4 Behavioral Tests:

Mice were kept in a breeding room and tested in that same room under guidelines as evaluated by AALAC. The University of Illinois Chicago Animal Care Committee approved all experiments. For all of the tests described below, mice between 60-120 days old were used except for the 3 arm maze, delayed

spontaneous alternation maze and open field where mice were 90-120 days old.

Mice were also all naïve to the maze prior to the first test. Unless otherwise

stated, ten mice from each sex and genotype were used.

2.4.1 Social Preference Test

Five mice from each sex and genotype were used for this experiment for a

total of 20 mice. The maze was a Y-maze with two tethers in two of the arms

(Figure 2). A male and a female *SnJ* mouse between 150-200 days old were

tethered in separate arms with a zip-cord around their middle. The tethered mice

were unable to leave their respective arms. A test mouse was placed in the

vacant arm and its interactions with the other two mice were recorded for 30

minutes. Time in contact with the tethered mouse (touching, fighting etc.) was

quantified as well as percent time in the arm with the tethered animals.
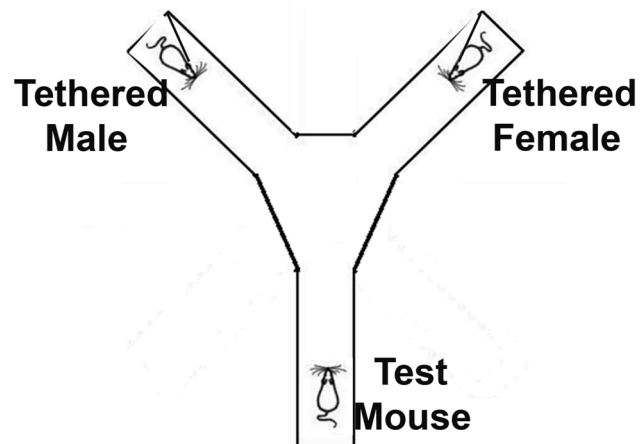
Figure 2 Social Preference Task.

A diagram of the social preference task. The two tethered mice are confined to their arms of the maze while the test mouse is able to move between all of the arms.

2.4.2 Four Arm Spontaneous Alternation Task

The maze consists of four arms of equal length that are equal distance from the center of the maze. The animals were given ten minutes to explore the maze and each arm entry was recorded. An arm entry was only considered complete when all four paws crossed fully into the arm. The animals were positioned randomly into the center of the maze to start the task. An animal was considered to have made an alternation when they entered four different arms consecutively. Every set of four consecutive choices were considered an alternation (see example Figure 3 (Lalonde, 2002)). Therefore one alternation could be entering arms A, B, D, and then C. The animal could continue to alternate if it then entered arm A again, however would cease to alternate if it then entered arm D afterwards, for a total chain of entries of A, B, D, C, A, D. An alternation score was then calculated, which is the number of alternations divided by the number of total possible alternations. In this example the animal would have alternated twice which would be divided by three possible alternations, for a score of 67%. The total number of arm entries and percent alternation were recorded for each mouse. All experiments were recorded with a video camera for later confirmation of arm entries and alternations.
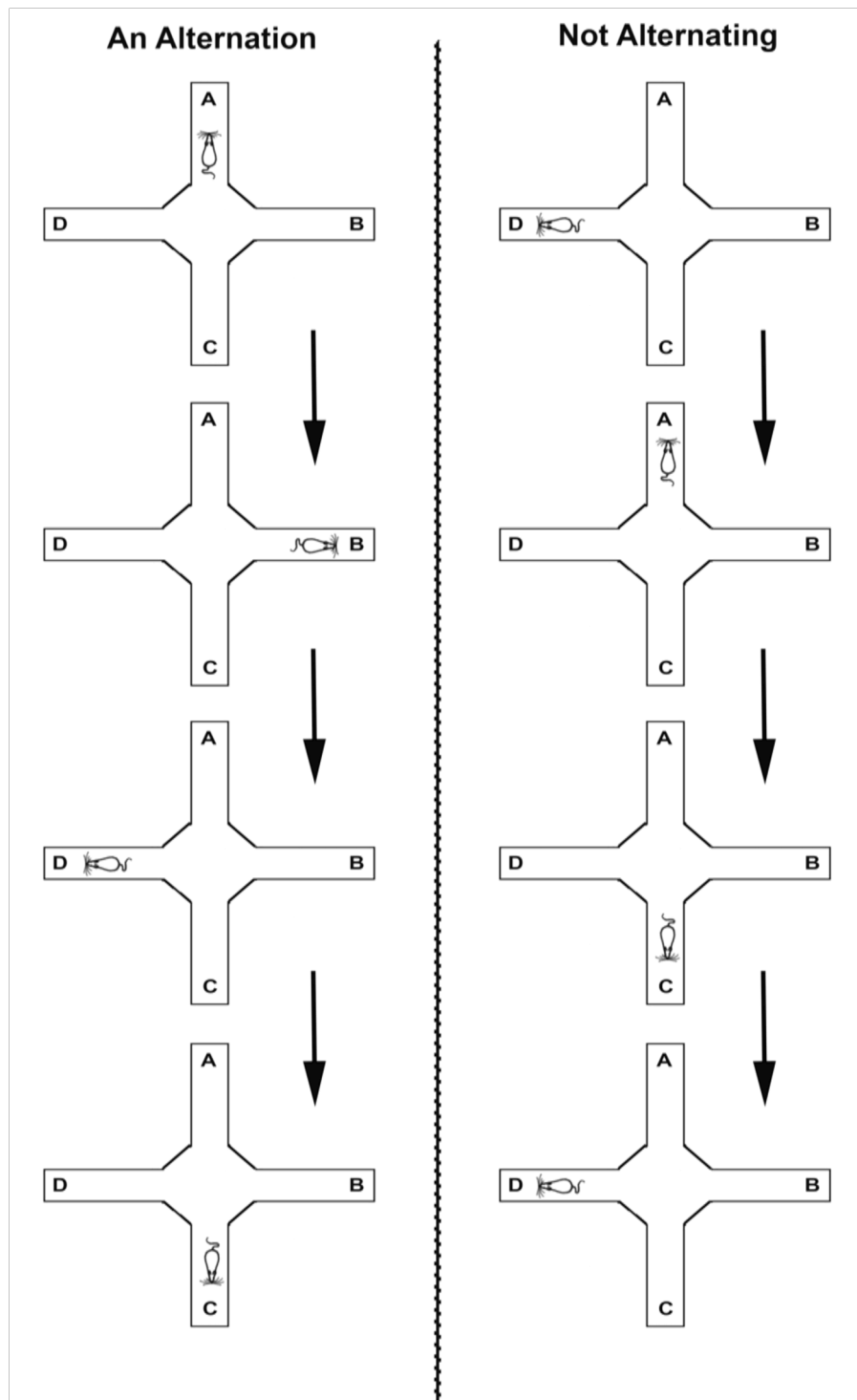
Figure 3 An example of spontaneous alternation.

The left is showing an animal that successfully alternates once, whereas the animal on the right fails to alternate.

2.4.3 Estrus Four-arm maze experiment:

Female mice were examined at the four different stages of the estrus cycle prior to testing in the four-arm spontaneous alternation task as described above. At least ten mice were tested per stage of estrus for the *sut*, *SnJ*, *B6* and *xCT* genotypes for a total of over 160 mice.   A vaginal smear was taken prior to testing and then later examined using a light microscope. Estrus staging was then confirmed using the Pap smear technique described in (Hubscher et al., 2005). Briefly, slides were washed in 95% ethanol for five minutes. They were then dipped ten times in distilled water, followed by two minutes in Gill's Hematoxylin (Fisher Scientific). They were then rinsed again in distilled water for ten dips followed by one minute in Scott's Tap water (Sigma-Aldrich). The slides were rinsed again for ten dips in distilled water followed by ten dips in 95% ethanol. They were then placed in Orange G6 (Lerner Laboratories) for one minute, followed by ten dips in 95% ethanol. The slides were then placed in Eosin-Azure 50 (Lerner Laboratories) for ten minutes and rinsed for 20 dips in 95% followed by 10 dips in 100% ethanol. They were then lastly dipped ten times in Xylene (Fisher Scientific) and then allowed to dry before examination. Estrus stage was determined by the ratio of nucleated and cornified cells and leukocytes present in the vaginal smear (Figure 4).

2.4.4 Plus Arm Anxiety Maze:

The maze consisted of four arms, two of which were open and did not have walls, and two of which with walls. The maze was suspended 60cm above

the floor of the testing room, such that in the open arms, the mouse could look over the side of the arm to the floor below. Mice were placed in the center of the maze and allowed to explore while their arm choices were recorded. Mice were tested for ten minutes a day for three consecutive days. The proportion of time spent in the open arms versus the closed, as well as number of open/closed arm choices and total number of arm entries were recorded for this task (Figure 5 (Crawley, 2008)).
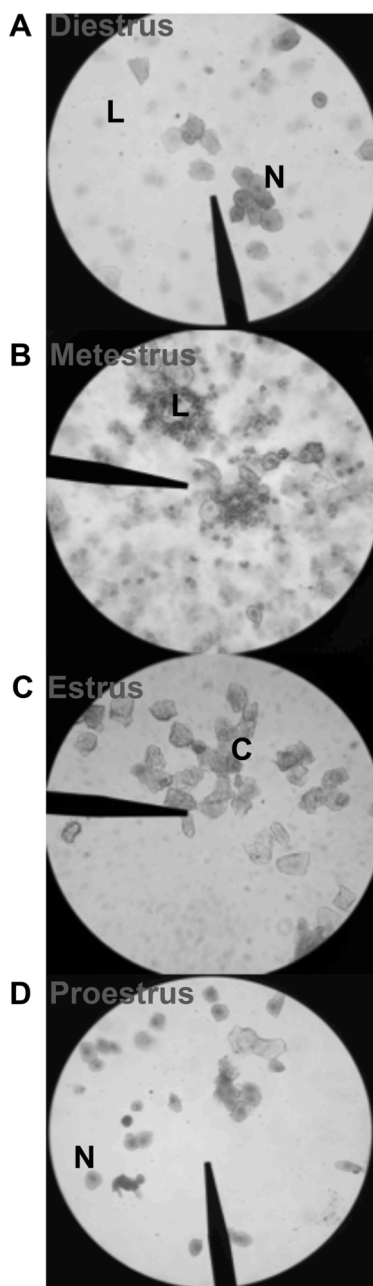
Figure 4 Examples of estrus stage results from female mice.

A, diestrus, a stage that an even mix between leukocytes (L) and nucleated cells (N), B, metestrus, stage that is predominantly leukocytes (L) with some nucleated cells and cornified cells (C). C, estrus, stage is mostly cornified cells (C). D, proestrus, mostly nucleated cells (N).

Figure 5 Plus arm anxiety maze diagram.

Shaded arms indicate where the walls of the plus arm maze were taken away. The maze was suspended 60cm from the ground and mice could look over the edge in the shaded arms.
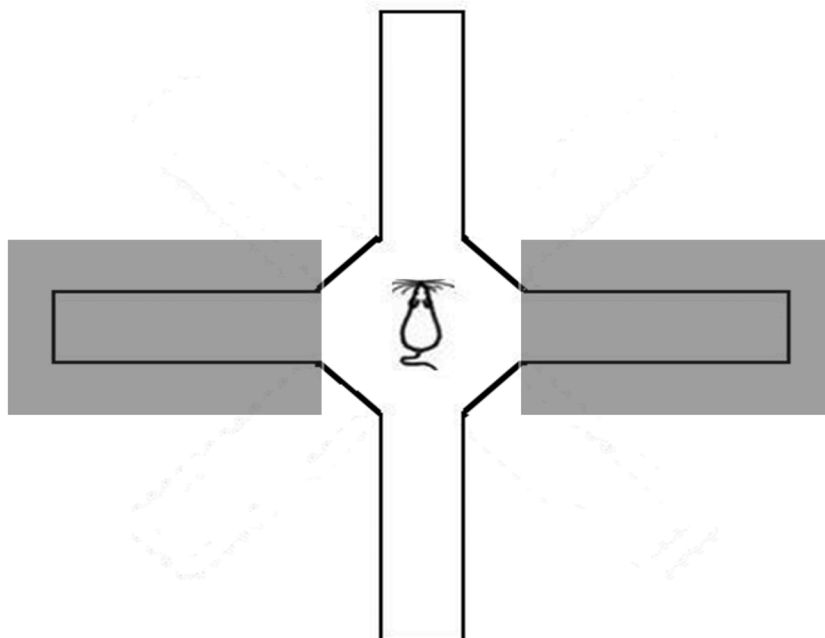
2.4.5 8 Arm Memory Task:

The maze consisted of eight arms each of which had a fruity pebble placed in a small well at the end of the arm (Figure 6). Mice were food deprived to 85% of their body weight prior to testing. Mice were also given fruity pebbles prior to testing to acclimate them to the novel food. The total number of arm choices it took for the mouse to eat all eight fruity pebbles was recorded for each trial over ten trials. An entry was only considered if the mouse got all four paws into the arm. Two trials took place a day, one in the morning and one in the afternoon. The number of errors was also recorded, an entry was considered an error if the mouse had already eaten the fruity pebble that was in that arm (Crawley, 2008).

2.4.6 Rotarod Task:

Mice were naïve to this task, however most were also used in the four-arm spontaneous alternation task. A mouse was placed on a rotarod (Accuscan Instruments), which is a motorized rod that continues to increase in speed as the amount of time the mouse spends on the rod increases until eventually it falls off (JONES and ROBERTS, 1968). The test lasts for 5 minutes (300 seconds) and can get up to 40 rpms or when the mouse falls off. Speed the rod has reached and amount of time spent on the rod are recorded for each mouse as they fall off, each mouse is given five trials in a day and the average over the day for those trials was quantified. Mice were tested for six consecutive days (Figure 7).
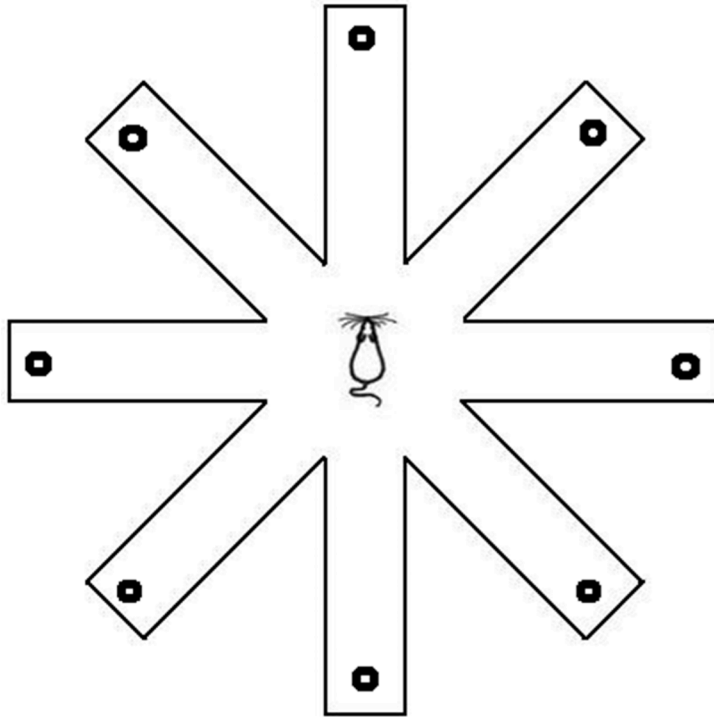
Figure 6 8 arm memory maze diagram.

A diagram of the 8 arm memory maze. Circles at the end of arms contain fruity pebbles.

Figure 7 Rotarod Task

2.4.7 Delayed Spontaneous Alternation Task:

Mice between 90-120 days old were used for this experiment. For female mice, a vaginal smear slide was taken on the day of testing to determine the stage of estrus followed later by pap staining for confirmation. A mouse was placed into the start arm (the stem of the Y, arm F2) of a three-arm Y-maze with one of two arms blocked off (the top of the Y, arm N), and was allowed to explore for 15 minutes. The mouse was then placed in a neutral cage for five minutes. The mouse was then reintroduced into the start arm of the Y-maze but allowed to explore all three arms for five minutes. Time spent in each arm, number of arm choices and first arm choice (novel arm, versus non-novel arm) were recorded for each mouse. An arm entry was only considered complete if all four of the mouse's paws completely entered the arm. The last portion of this experiment (the five minute testing phase) was video-recorded for each mouse for later analysis and confirmation of testing (Figure 8).
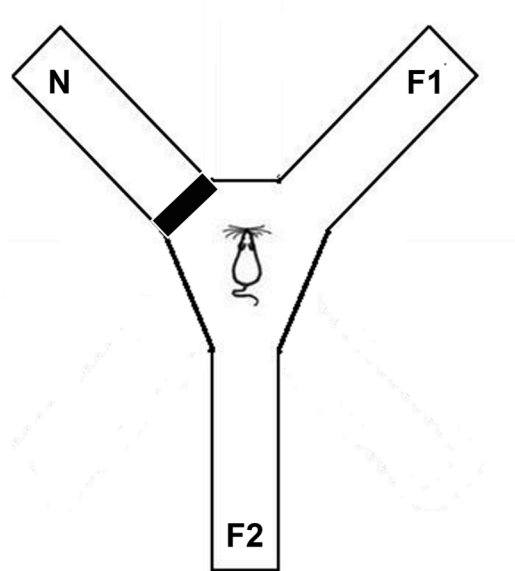
Figure 8 Delayed spontaneous alternation maze.

Mice were exposed to this maze for 15 minutes with the novel arm being blocked off. Mice were then removed from the maze for five minutes. The test started when the mice were then returned to the maze for five minutes with access to the open novel arm. Time spent in each arm and arm choice were recorded.

2.4.8 Open-Field Task:

Mice between the ages of 90-120 days old were used in this experiment. A total of ten mice for each sex, genotype and their controls were used in this experiment for a total of 120 mice. For female mice, a vaginal smear slide was taken on the day of testing to determine the stage of estrus followed later by pap staining for confirmation. All mice used in this maze were naïve to the maze; however they were also used in the three-arm spontaneous alternation task as well. The maze consisted of an opaque/clear frog tank that had been sectioned into nine sections, with a middle square (Figure 9 (Denenberg and Morton, 1962)). All of the sections were of roughly equal size. A mouse was placed in the middle space to start and was allowed to explore the maze for eight minutes. Number of sections crossed and proportion of time spent in the middle section were quantified for each mouse. All mice were taped using a video camera for later comparison and quantification of results.

2.4.9 Three-Arm Spontaneous Alternation Task

Mice between the ages of 90-120 days old were used in this experiment. A total of ten mice for each sex, genotype and their controls were used in this experiment for a total of 120 mice. For female mice, a vaginal smear slide was taken on the day of testing to determine the stage of estrus followed later by pap staining for confirmation. All mice used in this maze were naïve to the maze; however they were also used in the open-field maze. The maze had three arms, which the animal was allowed to explore freely for eight minutes (Lalonde, 2002).

The number of arm entries and order of arm entries was recorded to compute a spontaneous alternation percent. Every set of three consecutive novel arm choices could be considered an alternation. Therefore one alternation could be entering arms A, C, and then B. The animal could continue to alternate if it then entered arm A again, however would cease to alternate if it then entered arm B afterwards, for a total chain of entries of A, C, B, A, B. An alternation score was then calculated, which is the number of alternations divided by the number of total possible alternations. In this example the animal would have alternated twice which would be divided by three possible alternations, for a score of 67%. The total number of arm entries and percent alternation were recorded for each mouse. All experiments were recorded with a video camera for later confirmation of arm entries and alternations.
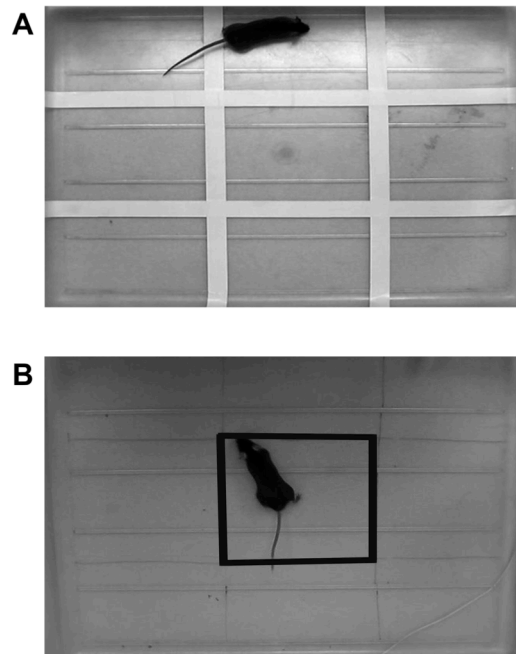
Figure 9 Open field maze diagram.

A, an example of a mouse exploring the maze. Number of grid crossings is quantified. B, an example of a mouse in the middle portion of the maze.

2.5 Sulfasalazine Experiments:

16 mice from each sex, genotype and their controls were used for this experiment, only *sut* and *SnJ* were tested for a total of 64 mice. Eight mice of each sex and genotype were randomly assigned either saline or Sulfasalazine (SAS, Sigma-Aldrich). All of the animals were tested on the rotarod and four-arm spontaneous alternation task (described previously) as a baseline prior to injections. All of the animals were naïve to these tasks prior to testing. The next day they were either injected I.P. with a solution of 8mg/mL SAS or 1mL saline and tested again in the rotarod and four-arm maze, this was considered an acute measure of the drug's effectiveness. The animals were then given twice-daily I.P. injections of SAS or saline for a total of seven days. This was similar to the regimen performed by Chung et al., 2005 which showed an effect of SAS on gliomas in mice. On the seventh day, after the last injection, animals were tested again in the rotarod and four-arm task; this was considered a measure of chronic SAS exposure. Animals were tested within 30 minutes of injection to ensure that the drug had not been metabolized (Figure 9).

Figure 10 Sulfasalazine dose regimen

Baseline Tests:
Rotarod
4 Arm Spon Alt

Day 0

Acute:
1 injection
Rotarod
4 Arm Spon Alt

Day 1

2x Daily Injections 8mg/mL
SAS or Saline

Chronic:
14 injections
total
Rotarod
4 Arm Spon Alt

Day 7
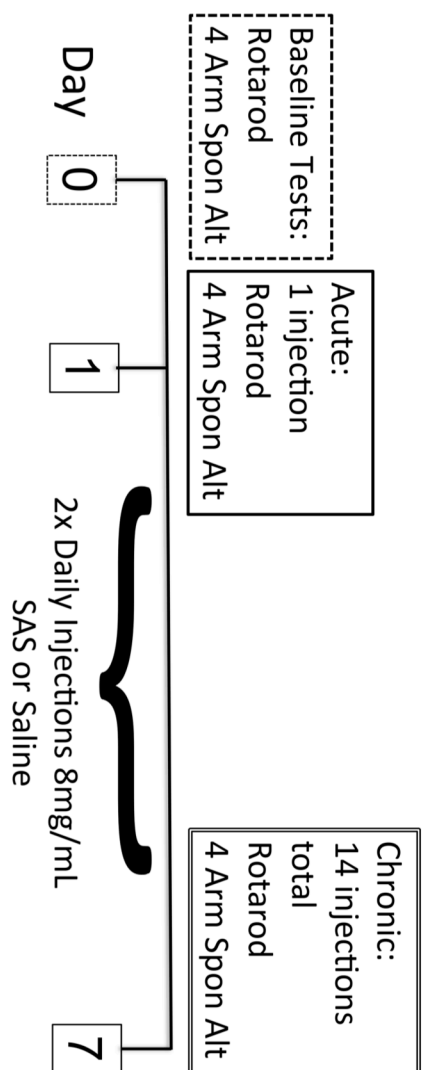
3 *SUT* MUTANT BEHAVIORAL ANALYSIS

3.1 Introduction

The *sut* mutation was one of the first characterized mouse mutants of system xc- (Chintala et al., 2005). *sut* mice should have behavioral changes due to loss of system xc- through loss of cystine transport and therefore cell death or loss of glutamate extrusion resulting in changes in synaptic strength through changes in ionotropic or metabotropic receptors.

Two main types of experiments were used to analyze changes in *sut* mouse behavior, a social preference test and a working memory task. The social preference task was modified from a protocol established by Williams et al to elucidate if *sut* mice also have modified social cues similar to what was seen in *genderblind Drosophila* (Williams et al., 1992). The spontaneous alternation task was used to determine if *sut* mice had altered spatial working memory similar to what was seen in *xCT* mice (De Bundel et al., 2011). The spontaneous alternation task uses a mouse's natural inclination to explore a novel environment and measures their efficiency in exploring the maze (Lalonde, 2002).

Based on the results from these two tasks, additional behavioral tests were analyzed to determine the nature of the deficits in the *sut* mice. The expected results were that *sut* mice would have impaired social interactions and

impaired ability to explore a four-arm spontaneous alternation maze compared to their genetic background *SnJ*.

3.2 Results

3.2.1 Social Preference Task

Drosophila system xc- knockout flies had an interesting bisexual behavioral phenotype (Grosjean et al., 2007). A social preference task was used to assess if *sut* animals had altered social behavior compared to their controls. Figure 1 shows that *sut* male test mice spent the same amount of time as their controls *SnJ* in arms containing both male and female mice (Two-way ANOVA with Bonferroni posttest, seconds spent in male arm *sut* 910.8 ± 137 seconds, n=4, *SnJ* 941.2 ± 97 seconds, n=5, p>0.05; female arm *sut* 493.8 ± 96 seconds, n=4, *SnJ* 401.6 ± 77.4 seconds, n=5, p>0.05, Figure 1B). However, both *sut* and *SnJ* mice spent significantly less time in the female arm (Comparison between the male and female arms, t-test of combined means for *sut* and *SnJ* p<0.01 Figure 1B). *sut* and *SnJ* males also spent the same amount of time in contact with the male and female mice in the arms; both genotypes spent significantly more time with the male mice than the females (Two-way ANOVA with Bonferroni posttest, percent time in contact with male mouse, *sut* 51.5 ± 12.2%, n=4, *SnJ* 64.0 ± 7.2%, n=5, p>0.05; percent time in contact with female mouse *sut* 30.7 ± 10.6%, n=4, *SnJ* 27.1 ± 11.3%, n=5, p>0.05, Figure 1C. Comparison

between the male and female arms, t-test of combined means for *sut* and *SnJ* p<0.05).

Female *sut* mice also did not have any aberrant social behaviors compared to their controls. Both *SnJ* and *sut* mice spent similar amounts of time in the male and female arms, with both genotypes spending significantly more time in the male arm (Two-way ANOVA with Bonferroni posttest, seconds spent in male arm *sut* 877.2 ± 249.3 seconds, n=5, *SnJ* 1003.0 ± 185.5 seconds, n=5, p>0.05; female arm *sut* 322.2 ±107.4 seconds, n=5, *SnJ* 309.2 ± 97.9 seconds, n=5, p>0.05, Figure 2B. Comparison between the male and female arms, t-test of combined means for *sut* and *SnJ* p<0.01). Female *sut* mice spent similar percent of time in contact with the male and female mouse in the arm; however, both genotypes spent significantly less time in contact with the female mouse (Two-way ANOVA with Bonferroni posttest, percent time in contact with male mouse, *sut* 41.3 ± 6.5%, n=5, *SnJ* 42.9 ± 7.9%, n=5, p>0.05; percent time in contact with female mouse *sut* 17.5 ± 7.9%, n=5, *SnJ* 19.0 ± 8.7%, n=5, p>0.05, Figure 2C. Comparison between the male and female arms, t-test of combined means for *sut* and *SnJ* p<0.001). Based on these results, neither male or female *sut* mice had an abnormal social preference compared to their controls as measured by this task.

A

Tethered Male

Tethered Female

Test Mouse

B Male Test Mice

C
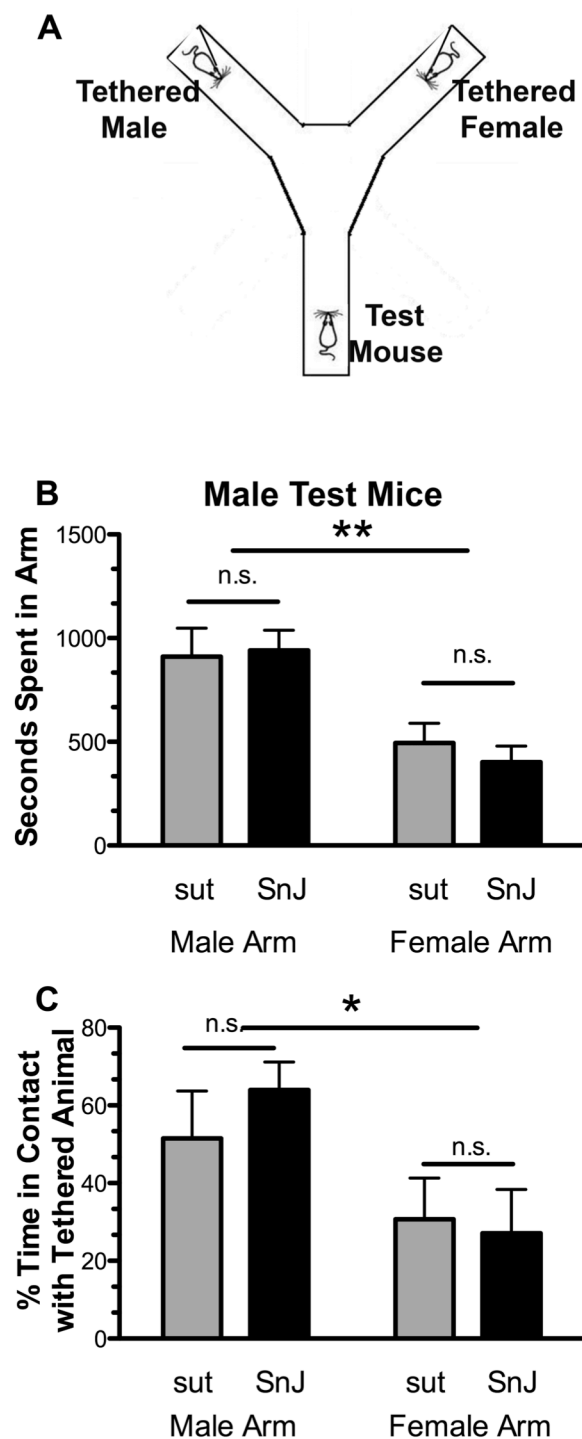
Figure 1 Social Preference Maze Task for Male *sut* Mice

A, A diagram showing the maze set up. B, C Results for experimental males in the maze showing seconds spent in the female or male arm (B) and % time in contact with the tethered animals (C) (n=4 or 5 per genotype for test animals)
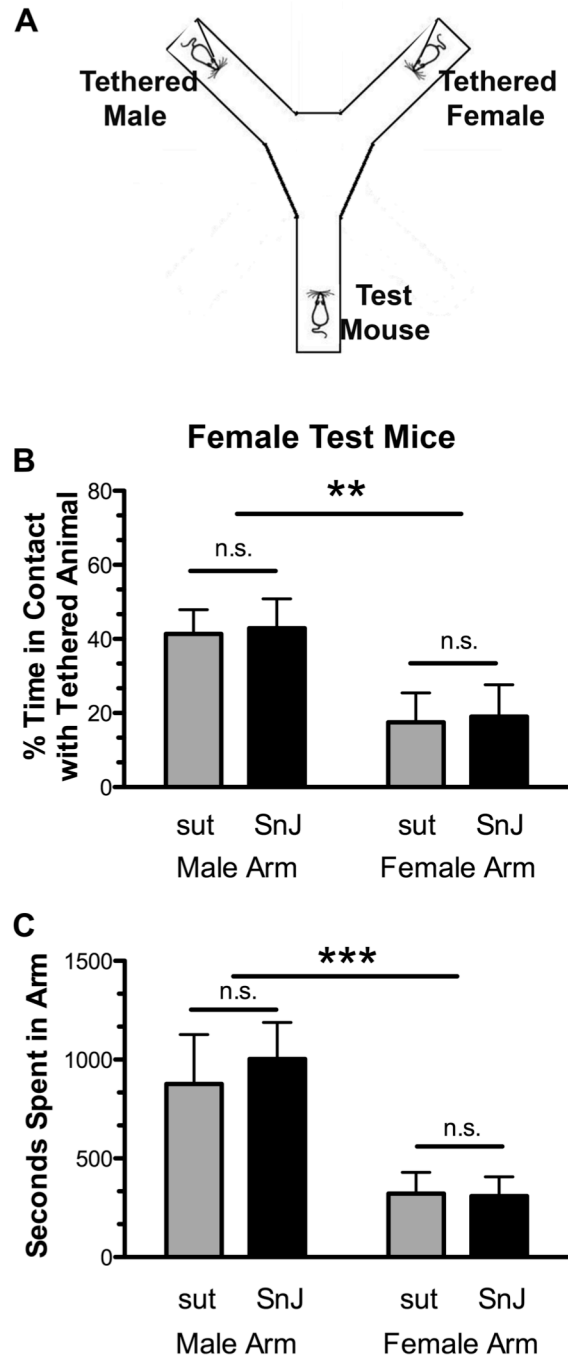
Figure 2 Social Preference Maze Task for *sut* Female Mice

A, A diagram showing the maze set up. B, C Results for experimental males in the maze showing seconds spent in the female or male arm (B) and % time in contact with the tethered animals (C) (n=5 for each genotype for test mice)

3.2.2 Four Arm Spontaneous Alternation Task

The abnormal sexual phenotype exhibited in *Drosophila* system xc-mutants may have been due to impairments in learning and memory (Grosjean et al., 2007). To test whether *sut* mice also had impairments in spatial working memory, they were tested in a four-arm version of the spontaneous alternation task. Male *sut* mice do not make fewer alternations compared to their controls, however they do make fewer arm choices (Two-tailed t-test of spontaneous alternation percent, *sut* 18.5 ± 4.9%, n=10, *SnJ* 20.6 ± 3.9% n=10, p=0.74, Figure 3B, Two-tailed t-test of number of arm choices, *sut* 19.9 ± 3.9 choices n=10, *SnJ* 32.7 ± 4.3 choices, n=10, p=0.04 Figure 3C). Female *sut* mice also had a reduced number of arm choices, but not percent alternations compared to their controls (Two-tailed t-test of spontaneous alternation percent, *sut* 12.2 ± 1.9%, n=69, *SnJ* 17.1 ± 1.9%, n=56 p=0.07, Figure 4B, Two-tailed t-test of number of arm choices, *sut* 13.0 ± 1.5 choices n=68, *SnJ* 19.8 ± 1.8 choices, n=57, p=0.004 Figure 4C). Based on these results, both male and female *sut* animals make fewer arm choices in a four-arm alternation maze, but do not make fewer alternations (though female *sut* mice have a trend towards significance).
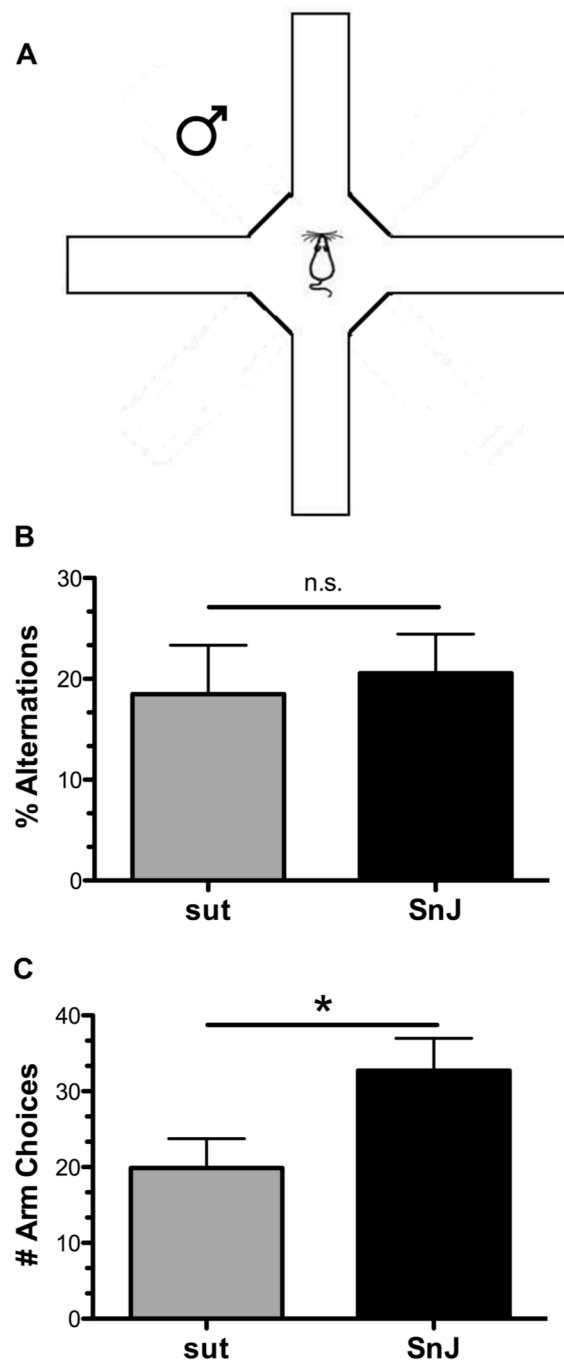
Figure 3 Four Arm Spontaneous Alternation Maze for *sut* males

A, a diagram of the maze, B % alternations compared to controls, C, number of arm choices compared to controls (n=10 for each genotype) *=p<0.05

Figure 4 Four Arm Spontaneous Alternation Maze for *sut* females

A, a diagram of the maze, B % alternations compared to controls, C, number of arm choices compared to controls **p<0.01
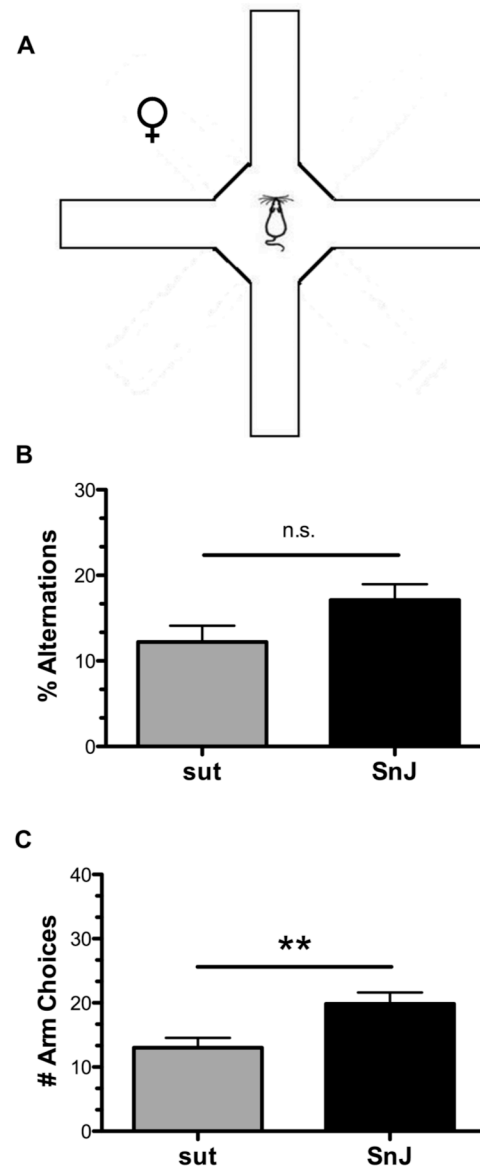
3.2.3 Plus Arm Anxiety Maze

There are several hypotheses as to why *sut* mice might make fewer arm choices than controls. One of which is that *sut* mice tend to have more anxiety than *SnJ* animals and therefore do not explore the maze as much. To test this, *sut* mice were tested in a plus-arm anxiety maze composed of two open arms (without walls) and two closed arms (with walls). Animals that tend to spend more time in open arms are thought to have less anxiety than animals that spend most of their time in closed arms (Crawley, 2008). Over three days, *sut* mice did not spend significantly less time in open arms than *SnJ* animals (Two-way ANOVA with Bonferroni posttest, percent time in open arms, *sut* day 1 31.0 ± 8.5%, n=10, *SnJ* 31.3 ± 5.5%, n=10, p>0.05 day 2 *sut* 9.7 ± 4.7%, n=10, *SnJ* 7.5 ± 3.1%, n=10, p>0.05, day 3 *sut* 5.1 ± 2.1% n=10, *SnJ* 6.2 ± 2.5%, n=10, p>0.05 Figure 5B). *sut* males also made the same proportion of open arm to closed arms choices compared to controls (Two-way ANOVA with Bonferroni posttest, proportion of open arm choices, *sut* day 1 50.8 ± 8.8 choices, n=10, *SnJ* 52.1 ± 5.0 choices, n=10, p>0.05 day 2 *sut* 30.1 ± 9.7 choices, n=10, *SnJ* 25.5 ± 7.1 choices, n=10, p>0.05, day 3 *sut* 15.0 ± 6.8 choices n=10, *SnJ* 16.0 ± 5.3 choices, n=10, p>0.05 Figure 5C). *sut* males did not make fewer total arm choices than their controls (Two-way ANOVA with Bonferroni posttest, total number of arm choices, *sut* day 1 7.4 ± 1.5 choices, n=10, *SnJ* 11.8 ± 1.1 choices, n=10, p>0.05 day 2 *sut* 6.2 ± 1.1 choices, n=10, *SnJ* 6.9 ± 1.4 choices, n=10, p>0.05, day 3 *sut* 4.9 ± 0.9 choices n=10, *SnJ* 8.6 ± 2.1 choices, n=10,

p>0.05 Figure 5D). Based on these results, male *sut* mice do not appear to have an anxiety phenotype compared to their controls in this task.

Female *sut* mice also did not differ in the proportion of time spent in open arms compared to their controls (Two-way ANOVA with Bonferroni posttest, percent time in open arms, *sut* day 1 37.2 ± 11.1%, n=10, *SnJ* 28.4 ± 4.4%, n=10, p>0.05 day 2 *sut* 8.0 ± 3.3%, n=10, *SnJ* 13.5 ± 1.8%, n=10, p>0.05, day 3 *sut* 16.9 ± 11.0% n=10, *SnJ* 14.4 ± 4.5%, n=10, p>0.05 Figure 6B). They also did not make fewer open arm choices compared to their controls (Two-way ANOVA with Bonferroni posttest, proportion of open arm choices, *sut* day 1 50.0 ± 11.5 choices, n=10, *SnJ* 56.9 ± 6.5 choices, n=10, p>0.05 day 2 *sut* 31.2 ± 10.4 choices, n=10, *SnJ* 48.4 ± 2.8 choices, n=10, p>0.05, day 3 *sut* 12.9 ± 9.9 choices n=10, *SnJ* 39.8 ± 7.8 choices, n=10, p>0.05 Figure 6C). Female *sut* mice did make fewer total arm choices than their controls on all three days (Two-way ANOVA with Bonferroni posttest, total number of arm choices, *sut* day 1 5.9 ± 1.4 choices, n=10, *SnJ* 12.0 ± 0.9 choices, n=10, p<0.05 day 2 *sut* 3.5 ± 0.9 choices, n=10, *SnJ* 9.0 ± 1.4 choices, n=10, p<0.05, day 3 *sut* 3.7 ± 1.2 choices n=10, *SnJ* 12.3 ± 2.7 choices, n=10, p<0.001 Figure 6D). Female *sut* mice spent the same amount of time and made the same proportion of choices between open and closed arms compared to controls; however female *sut* mice made fewer arm choices total.   This indicates that the arm choice phenotype is not related to anxiety, however they still do make fewer arm choices compared to controls.

Figure 5 Plus Arm Anxiety Maze for Male *sut* mice.

A, a diagram of the maze (shaded areas are without walls), B, proportion of time spent in the open arms, C, number of choices that were open arms, D total number of arm entries.
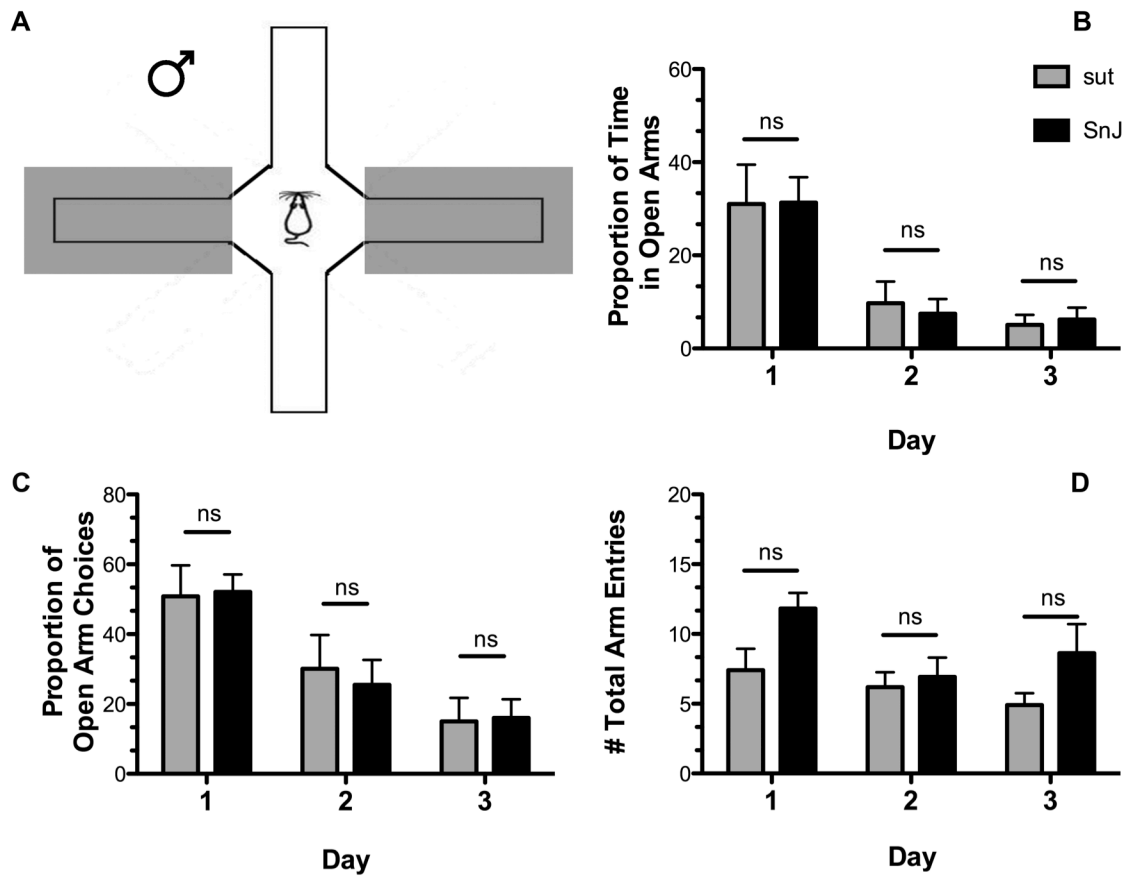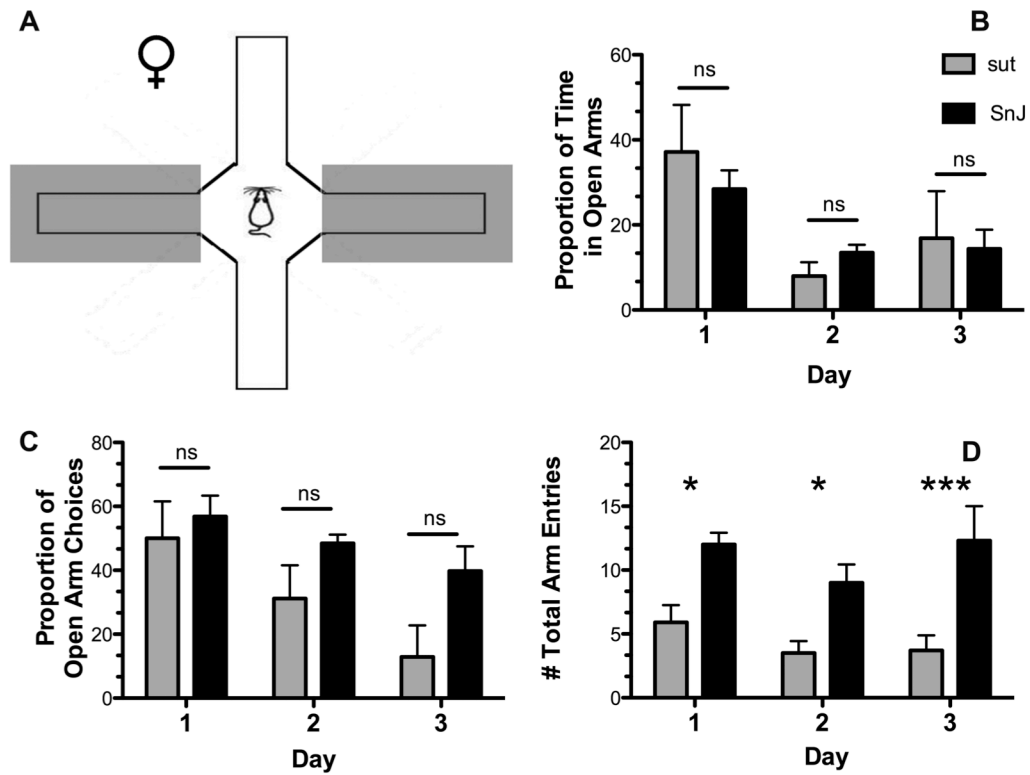
Figure 6 Plus Arm Anxiety Maze for Female *sut* mice.

A, a diagram of the maze (shaded areas are without walls), B, proportion of time spent in the open arms, C, number of choices that were open arms, D total number of arm entries (*=p<0.5, ***=p<0.001).

3.2.4 8 Arm Memory Maze

To test whether the *sut* arm choice phenotype was due to a memory deficit, an eight-arm memory task was performed. This test measures how many trials it takes for mice to find and consume eight food pellets that are distributed in the eight arms of a radial maze. This task measures spatial working memory to assess if mice can efficiently navigate a maze to find food reward (Crawley, 2008). *sut* males and females made the same number of arm choices in this task as controls (Two-way ANOVA with matching, male number of arm choices trial 1, *sut* 78.8 ± 13.7 choices n=5, *SnJ* 111.6 ± 14.3 choices n=5, p>0.05; trial 2 *sut* 57.4 ± 6.6 choices, n=5, *SnJ* 68.8 ± 5.9 choices, n=5, p>0.05; trial 3, *sut* 70.2 ± 4.2 choices, n=5, *SnJ* 46.0 ± 5.3 choices, n=5 p>0.05; trial 4, *sut* 48.6 ± 5.8 choices, n=5, *SnJ* 37.2 ± 8.5 choices, n=5 p>0.05, trial 5, *sut* 71.4 ± 5.6 choices, n=5, *SnJ* 53.4 ± 4.3 choices, n=5 p>0.05; trial 6, *sut* 32.8 ± 3.4 choices, n=5, *SnJ* 28.0 ± 5.7 choices, n=5 p>0.05; trial 7, *sut* 45.6 ± 7.6 choices, n=5, *SnJ* 43.4 ± 9.4 choices, n=5, p>0.05; trial 8, *sut* 24.2 ± 7.8 choices, n=5, *SnJ* 21.6 ± 4.0 choices, n=5, p>0.05; trial 9, *sut* 61.4 ± 12.1 choices, n=5, *SnJ* 41.0 ± 10.6 choices, n=5, p>0.05; trial 10, *sut* 52.4 ± 20.2 choices, n=5, *SnJ* 29.8 ± 5.0 choices, n=5 p>0.05, Figure 7B. Two-way ANOVA with matching, female number of arm choices trial 1, *sut* 72.6 ± 16.6 choices n=5, *SnJ* 65.2 ± 14.5 choices n=5, p>0.05; trial 2 *sut* 47.4 ± 12.4 choices, n=5, *SnJ* 40.2 ± 9.7 choices, n=5, p>0.05; trial 3, *sut* 73.0 ± 11.3 choices, n=5, *SnJ* 63.0 ± 10.8 choices, n=5 p>0.05; trial 4, *sut* 58.4 ± 10.6 choices, n=5, *SnJ* 36.0 ± 6.7 choices, n=5 p>0.05, trial 5, *sut* 79.8 ± 18.1 choices, n=5, *SnJ* 62.2 ± 10.2 choices, n=5 p>0.05; trial 6,

sut 44.2 ± 8.4 choices, n=5, *SnJ* 27.4 ± 3.2 choices, n=5 p>0.05; trial 7, *sut* 44.6

± 11.6 choices, n=5, *SnJ* 46.6 ± 13.8 choices, n=5, p>0.05; trial 8, *sut* 41.0 ± 10.5

choices, n=5, *SnJ* 28.8 ± 5.4 choices, n=5, p>0.05; trial 9, *sut* 32.8 ± 6.8 choices,

n=5, *SnJ* 48.4 ± 8.4 choices, n=5, p>0.05; trial 10, *sut* 32.8 ± 7.6 choices, n=5,

SnJ 34.0 ± 8.9 choices, n=5 p>0.05, Figure 7C). The arm choice phenotype

exhibited by *sut* males and females does not appear to be related to a memory

deficit as tested by the eight-arm memory maze.

A

B

C
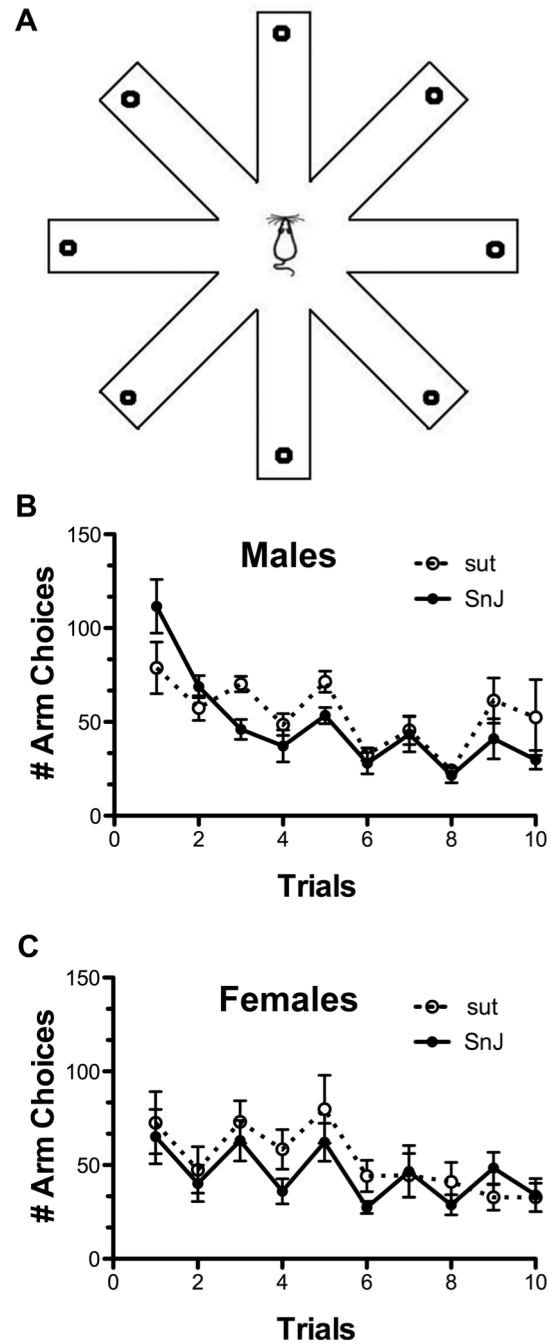
Figure 7 sut Mice Performance on an 8 Arm Memory Maze

A, a diagram of the maze, each arm had a fruity pebble in it B, sut male mouse number of arm choices before eating all 8 food pellets over 10 trials. C, sut female mouse number of arm choices before eating all 8 food pellets over 10 trials (n=10 animals per group).

3.2.5 Rotarod Task

To test whether the reduction in number of arm choices displayed by both male and female *sut* mice in the spontaneous alternation maze are due to a motor deficit, *sut* mice were tested in a rotarod task over six days. Neither male nor female *sut* mice displayed a motor coordination deficit compared to their controls in this task (Two-way ANOVA with matching fall time in seconds for males day 1, *sut* 54.2 ± 4.7 seconds n=10, *SnJ* 56.7 ± 6.8 seconds, n=10 p>0.05; day 2, *sut* 61.3 ± 6.2 seconds, n=10, *SnJ* 77.0 ± 8.8 seconds, n=10 p>0.05; day 3, *sut* 66.2 ± 10.4 seconds, n=10, *SnJ* 92.4 ± 7.4 seconds, n=10 p>0.05; day 4 *sut* 91.5 ± 9.3 seconds, n=10, *SnJ* 97.3 ± 7.4 seconds, n=10 p>0.05; day 5, *sut* 115.3 ± 11.3 seconds, n=10, *SnJ* 117.7 ± 9.9 seconds, n=10 p>0.05; day 6, *sut* 111.7 ± 11.1 seconds, n=10, *SnJ* 115.9 ± 8.2 seconds, n=10 p>0.05, Figure 8B. Two-way ANOVA with matching fall time in seconds for females day 1, *sut* 84.5 ± 6.8 seconds n=10, *SnJ* 75.1 ± 4.7 seconds, n=10 p>0.05; day 2, *sut* 76.3 ± 8.7 seconds, n=10, *SnJ* 113.7 ± 6.5 seconds, n=10 p>0.05; day 3, *sut* 97.5 ± 12.9 seconds, n=10, *SnJ* 132.6 ± 10.0 seconds, n=10 p>0.05; day 4 *sut* 129.7 ± 15.7 seconds, n=10, *SnJ* 150.0 ± 14.9 seconds, n=10 p>0.05; day 5, *sut* 140.2 ± 15.7 seconds, n=10, *SnJ* 177.5 ± 14.8 seconds, n=10 p>0.05; day 6, *sut* 150.2 ± 16.8 seconds, n=10, *SnJ* 192.3 ± 11.8 seconds, n=10 p>0.05, Figure 8C). The arm choice deficit exhibited by *sut* males and females appears to not be due to a motor coordination deficit as tested by this task.
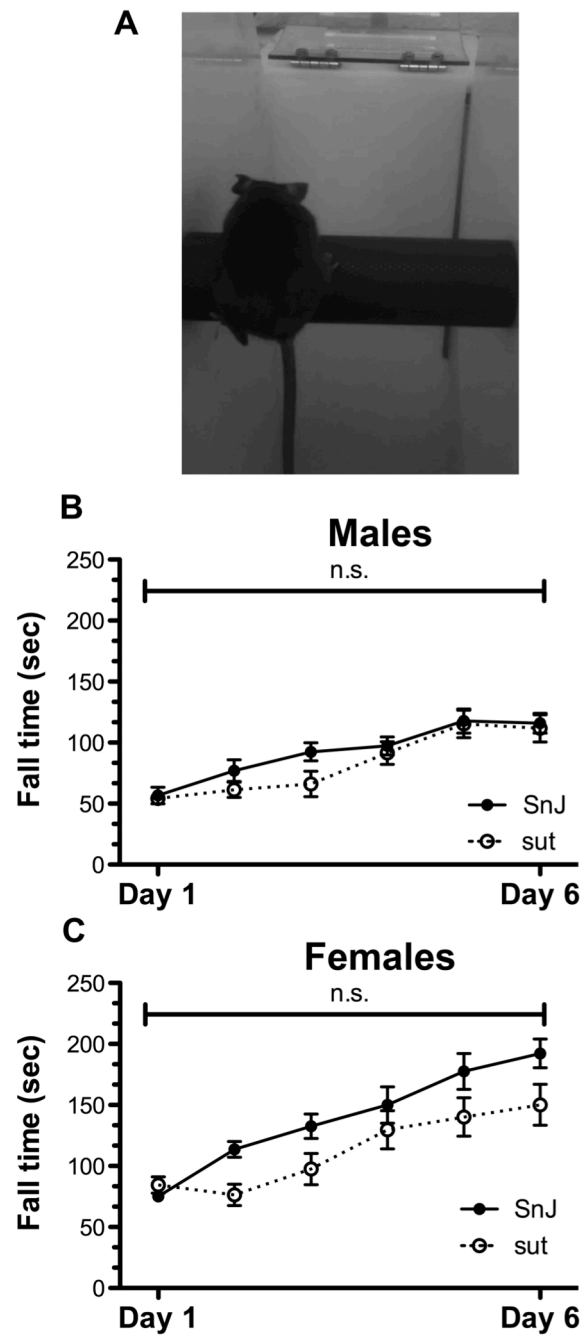
Figure 8 Male and Female *sut* Mutants Performance on a Rotarod Task Over Six Days.

A, an animal on the rotarod B, male fall time over six days C, female fall time over 6 days (n=10 for each genotype, none of the values are significant).

3.3 Discussion

In summary, *sut* system xc- knockout mice do not seem to have a social preference behavioral phenotype, however, both male and female *sut* mice make fewer arm choices than their controls in a four-arm spontaneous alternation task. This behavioral phenotype cannot be attributed to anxiety-related behavior, a memory deficit or reduced motor coordination ability, though *sut* females do continue to make fewer arm choices compared to controls in the anxiety maze.

While these results are interesting and give some insight into the potential role system xc- has on mammalian behavior, these deficits are very subtle in nature. These results are also consistent with the study by Li et al which showed impaired long-term memory in *sut* mice using a fear-conditioning behavioral assay (Li et al., 2012b). To further confirm if this behavioral phenotype can be attributed to system xc-, it would be useful to ascertain to if this behavioral phenotype can be reproduced pharmacologically with system xc- inhibitors.

4   SULFASALAZINE EXPERIMENTS

4.1 Introduction

System xc- knockout mice (*sut*) showed a reduction in the number of arm choices during a four-arm spontaneous alternation task compared to their controls (*SnJ*). One way to test if loss of system xc- accounts for this behavior is to give control mice a system xc- inhibitor and see if they have similar behavioral deficits as *sut* mice; specifically a reduction in number of arm choices in the four-arm spontaneous alternation task.

There are several known system xc- inhibitors; the two most common are sulfasalazine (SAS) and S-CPG. Unfortunately both have non-specific effects as well as being potent system xc- inhibitors (Patel et al., 2004). S-CPG is also a metabotropic receptor antagonist and SAS has a myriad of non-specific effects including some anti-inflammatory effects (Bedingfield et al., 1995; Ye et al., 1999). Since S-CPG's non-specific effects may directly impact synaptic signaling and therefore behavior, SAS was chosen as the inhibitor for these behavioral experiments.

SAS is known to be one of the most potent inhibitors of system xc- and has potential for crossing the blood brain barrier (Chung et al., 2005). I used the same dose that was shown in work of Dr. Sontheimer to reduce tumor size in patients with gliomas that have an increase in system xc- activity (Chung et al., 2005). Mice were tested behaviorally at baseline before any injection of SAS,

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acutely within an hour of SAS injection, and chronically after twice daily injections for seven days. Mice were tested in both the four-arm spontaneous alternation maze and the rotarod task. Based on the *sut* mutant mouse phenotype, it was hypothesized that *SnJ* mice would have normal number of spontaneous alternations and a reduced number of arm choices when given SAS either acutely or chronically compared to animals given saline.

4.2 Results

4.2.1 Male four-arm spontaneous alternation with sulfasalazine

As expected, *sut* male mice did not have any impairment in their spontaneous alternation ability when given SAS over the three time-points (baseline, acute and chronic) (Two-way ANOVA with matching percent alternation ability for *sut* male mice at baseline, saline $38.6 \pm 13.3\%$, n=7; SAS $12.6 \pm 4.5\%$, n=7 $p>0.05$; acute, saline $24.8 \pm 6.9\%$ n=7; SAS $15.3 \pm 7.7\%$, n=7 $p>0.05$; chronic, saline $19.7 \pm 6.9\%$ n=7, SAS $12.5 \pm 6.6\%$, n=7 $p>0.05$, Figure 1B). *SnJ* male mice given SAS did not have any impairment in spontaneous alternation ability compared to saline (Two-way ANOVA with matching percent alternation ability for *SnJ* male mice at baseline, saline $18.8 \pm 4.8\%$, n=8; SAS $30.9 \pm 6.0\%$, n=8 $p>0.05$; acute, saline $18.1 \pm 5.6\%$ n=8; SAS $23.3 \pm 6.2\%$, n=8 $p>0.05$; chronic, saline $20.9 \pm 4.9\%$ n=8, SAS $33.8 \pm 9.0\%$, n=8 $p>0.05$, Figure 1C).
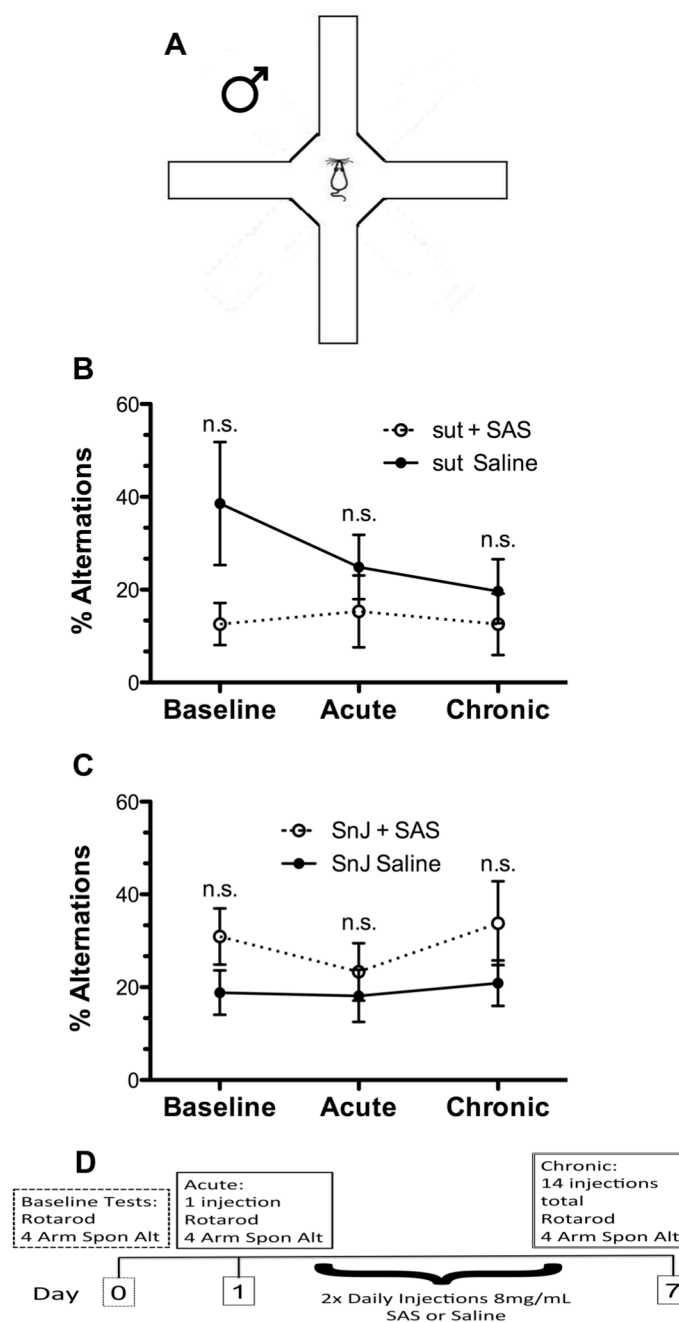
Figure 1, Male *sut* and *SnJ* mice percent spontaneous alternation ability in a four arm task with sulfasalazine or saline treatment.

A, diagram of the layout of the maze. B,C, % alternation for male *sut* mice (B) or SnJ mice (C) with SAS treatment or saline over three time periods, baseline (before any injections), acute (after the first injection), or chronic (after twice daily injections for seven days D, diagram of treatment schedule (n=7-8 for each treatment/genotype).

Male *sut* mice did not have any additional reduction in number of arm choices when given sulfasalazine compared to saline (Two-way ANOVA with matching number of arm choices for *sut* male mice at baseline, saline 16.1 ± 5.3 choices, n=7; SAS 22.4 ± 4.9 choices, n=7 p>0.05; acute, saline 18.7 ± 4.1 choices n=7; SAS 17.1 ± 5.3 choices, n=7 p>0.05; chronic, saline 24.3 ± 6.2 choices n=7, SAS 16.1 ± 5.5 choices, n=7 p>0.05, Figure 2B). SAS treatment of *SnJ* males did not result in a reduction in the number of arm choices compared to saline animals (Two-way ANOVA with matching number of arm choices for *SnJ* male mice at baseline, saline 16.4 ± 2.8 choices, n=8; SAS 24.0 ± 2.9 choices, n=8 p>0.05; acute, saline 15.4 ± 3.0 choices n=8; SAS 15.9 ± 3.2 choices, n=8 p>0.05; chronic, saline 23.6 ± 5.6 choices n=8, SAS 18.8 ± 3.3 choices, n=8 p>0.05, Figure 2C). Based on these data, SAS did not appear to impair spontaneous alternation ability, either through percent alternations or number of arm choices in either *sut* or *SnJ* males.

4.2.2 Female four-arm spontaneous alternation with sulfasalazine

As expected, *sut* female mice did not have any impairment in their spontaneous alternation ability when given sulfasalazine over the three timepoints (baseline, acute and chronic) (Two-way ANOVA with matching percent alternation ability for *sut* female mice at baseline, saline 13.9 ± 5.5%, n=8; SAS 8.6 ± 4.5%, n=8 p>0.05; acute, saline 18.8 ± 6.3% n=8; SAS 18.4 ± 6.0%, n=8 p>0.05; chronic, saline 27.8 ± 5.3% n=8, SAS 22.6 ± 6.1%, n=8 p>0.05, Figure 3B). *SnJ* female mice given SAS did not have any impairment in

spontaneous alternation ability compared to saline (Two-way ANOVA with matching percent alternation ability for *SnJ* female mice at baseline, saline 22.3 ± 3.1%, n=8; SAS 33.7 ± 3.8%, n=8 p>0.05; acute, saline 24.8 ± 4.9% n=8; SAS 30.5 ± 2.8%, n=8 p>0.05; chronic, saline 29.7 ± 3.7% n=8, SAS 30.5 ± 2.8%, n=8 p>0.05, Figure 3C).
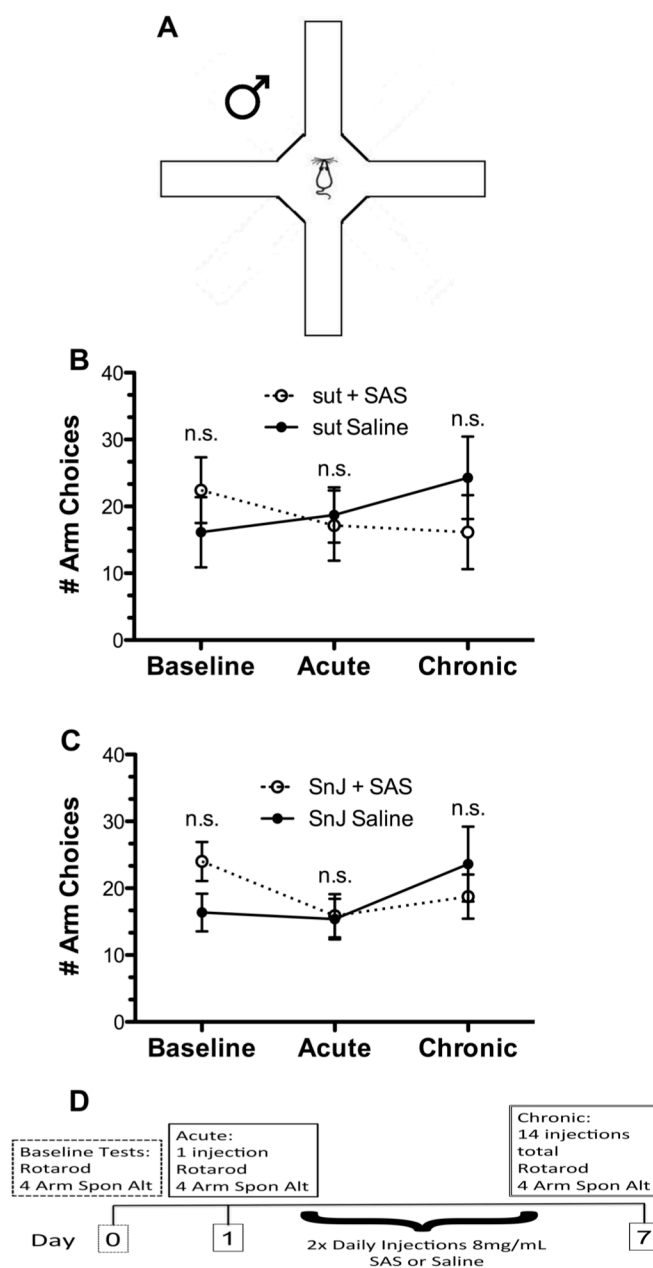
Figure 2, Male *sut* and *SnJ* mice number of arm choices in a four-arm task with sulfasalazine or saline treatment.

A, diagram of the layout of the maze. B,C number of arm choices for male *sut* mice (B) or SnJ mice (C) with SAS treatment or saline over three time periods, baseline (before any injections), acute (after the first injection), or chronic (after twice daily injections for seven days D, diagram of treatment schedule (n=7-8 for each treatment/genotype).
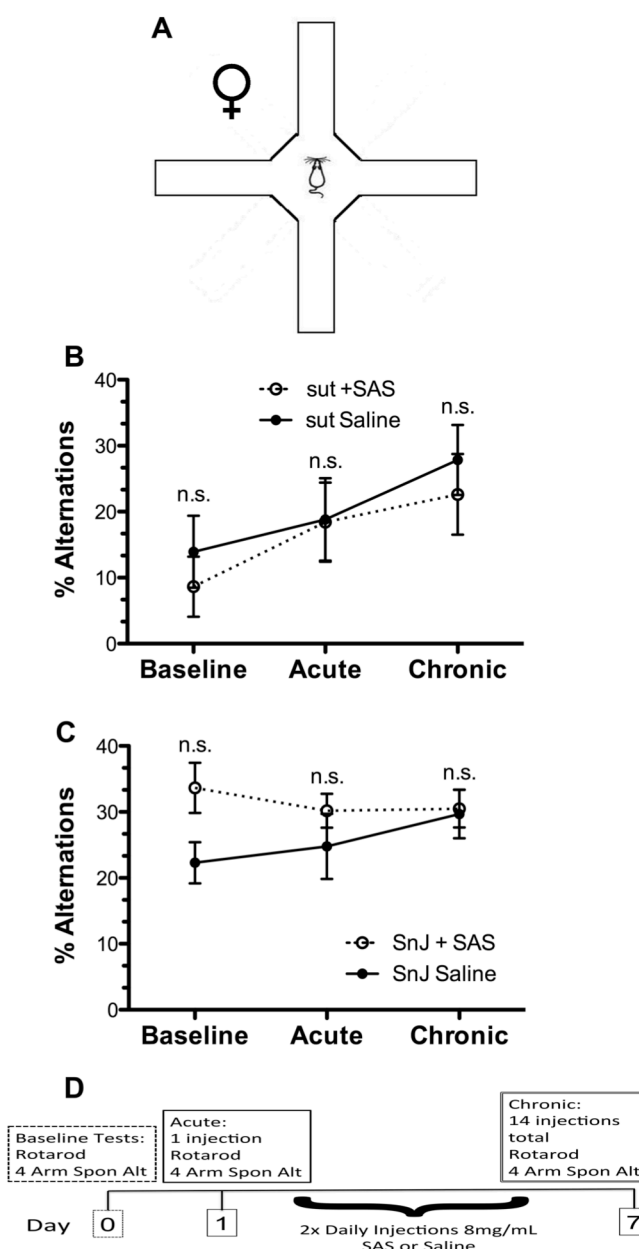
Figure 3, Female *sut* and *SnJ* mice percent alternations in a four-arm task with sulfasalazine or saline treatment.

A, diagram of the layout of the maze. B,C percent alternations for female *sut* mice (B) or *SnJ* mice (C) with SAS treatment or saline over three time periods, baseline (before any injections), acute (after the first injection), or chronic (after twice daily injections for seven days D, diagram of treatment schedule (n=7-8 for each treatment/genotype).

Female *sut* mice did not have any additional reduction in number of arm choices when given SAS compared to saline (Two-way ANOVA with matching number of arm choices for *sut* female mice at baseline, saline 12.0 ± 4.0 choices, n=8; SAS 17.3 ± 5.7 choices, n=8 p>0.05; acute, saline 16.3 ± 4.4 choices n=8; SAS 18.1 ± 5.6 choices, n=8 p>0.05; chronic, saline 21.5 ± 4.0 choices n=8, SAS 22.5 ± 5.6 choices, n=8 p>0.05, Figure 4B). SAS treatment of *SnJ* females did not result in a reduction in the number of arm choices compared to saline animals (Two-way ANOVA with matching number of arm choices for *SnJ* female mice at baseline, saline 27.6 ± 1.6 choices, n=8; SAS 27.7 ± 3.1 choices, n=8 p>0.05; acute, saline 32.5 ± 5.1 choices n=8; SAS 30.1 ± 3.2 choices, n=8 p>0.05; chronic, saline 27.3 ± 3.6 choices n=8, SAS 26.4 ± 4.0 choices, n=8 p>0.05, Figure 4C). Thus, SAS did not appear to impair spontaneous alternation ability in either *sut* or *SnJ* females for both percent alternations and number of arm choices.

4.2.3 Male rotarod performance with sulfasalazine

As a control for motor ability, *sut* and *SnJ* mice were also tested on a rotarod with either SAS or saline treatment. Male *sut* mice given SAS did not have any impairment in the rotarod task across the three time points compared to animals given saline (Two-way ANOVA with matching of fall time in seconds for *sut* male mice at baseline, saline 23.9 ± 2.5 seconds, n=8, SAS, 13.9 ± 1.2 seconds, n=8 p>0.05; acute, saline 35.9 ± 2.4 seconds, n=8, SAS 31.4 ± 5.5 seconds, n=8, p>0.05; chronic, saline 47.2 ± 7.5 seconds, SAS 37.9 ± 7.6

seconds, n=8, p>0.05, Figure 5B). Male *SnJ* mice given SAS also did not have any impairment in the rotarod task compared to animals given saline (Two-way ANOVA with matching of fall time in seconds for *SnJ* male mice at baseline, saline 39.2 ± 9.5 seconds, n=8, SAS, 38.5 ± 2.9 seconds, n=8 p>0.05; acute, saline 53.2 ± 8.4 seconds, n=8, SAS 54.3 ± 6.8 seconds, n=8, p>0.05; chronic, saline 75.1 ± 10.8 seconds, SAS 70.7 ± 9.2 seconds, n=8, p>0.05, Figure 5C). These data indicate, SAS treatment did not lead to impairment in the rotarod task in either *SnJ* or *sut* males.

4.2.4 Female rotarod performance with sulfasalazine

Female *sut* mice given SAS did not have any impairment in the rotarod task across the three time points compared to animals given saline (Two-way ANOVA with matching of fall time in seconds for *sut* female mice at baseline, saline 28.3 ± 4.7 seconds, n=8, SAS, 38.2 ± 4.4 seconds, n=8 p>0.05; acute, saline 43.2 ± 7.3 seconds, n=8, SAS 48.8 ± 6.5 seconds, n=8, p>0.05; chronic, saline 50.7 ± 7.6 seconds, SAS 43.5 ± 5.5 seconds, n=8, p>0.05, Figure 6B). Female *SnJ* mice given SAS also did not have any impairment in the rotarod task compared to animals given saline (Two-way ANOVA with matching of fall time in seconds for *SnJ* female mice at baseline, saline 46.1 ± 6.4 seconds, n=8, SAS, 50.8 ± 6.2 seconds, n=8 p>0.05; acute, saline 65.7 ± 10.8 seconds, n=8, SAS 76.3 ± 10.3 seconds, n=8, p>0.05; chronic, saline 84.4 ± 8.5 seconds, SAS 76.3 ± 10.3 seconds, n=8, p>0.05, Figure 6C). There results suggest that, SAS

treatment did not lead to impairment in the rotarod task in either *SnJ* or *sut*

females.
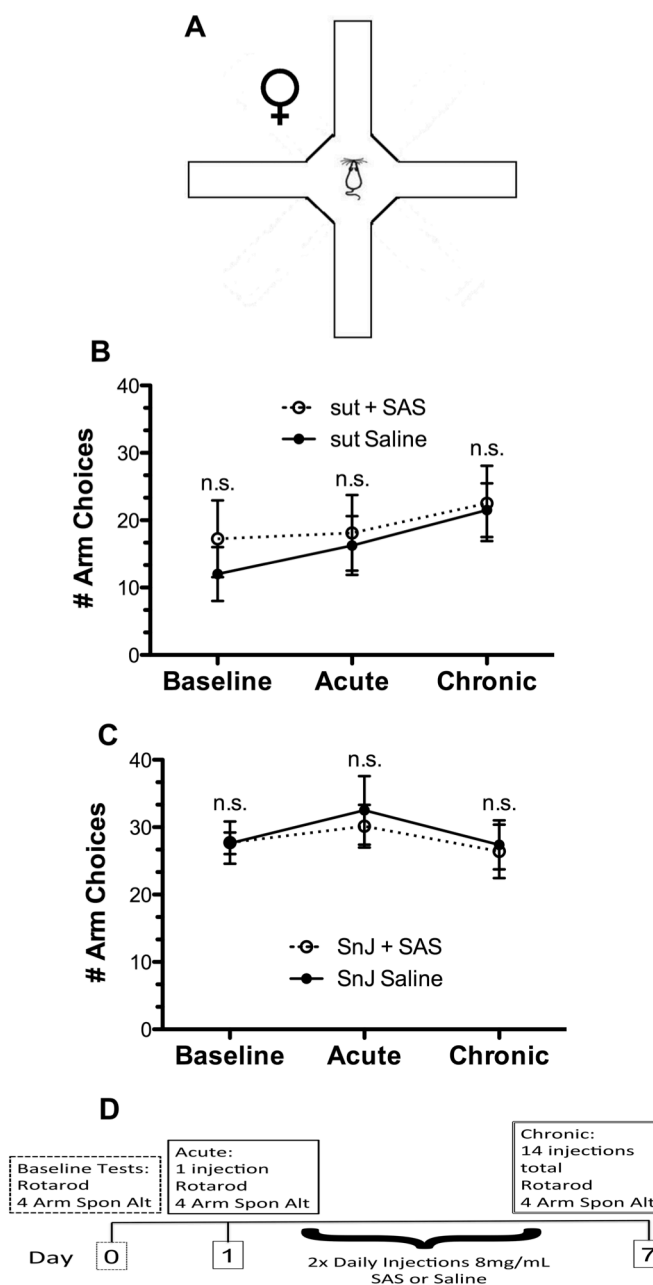
Figure 4, Female *sut* and SnJ mice performance in a four-arm task with sulfasalazine or saline treatment.

A, diagram of the layout of the maze. B,C number of arm choices for female *sut* mice (B) or SnJ mice (C) with SAS treatment or saline over three time periods, baseline (before any injections), acute (after the first injection), or chronic (after twice daily injections for seven days D, diagram of treatment schedule (n=7-8 for each treatment/genotype).
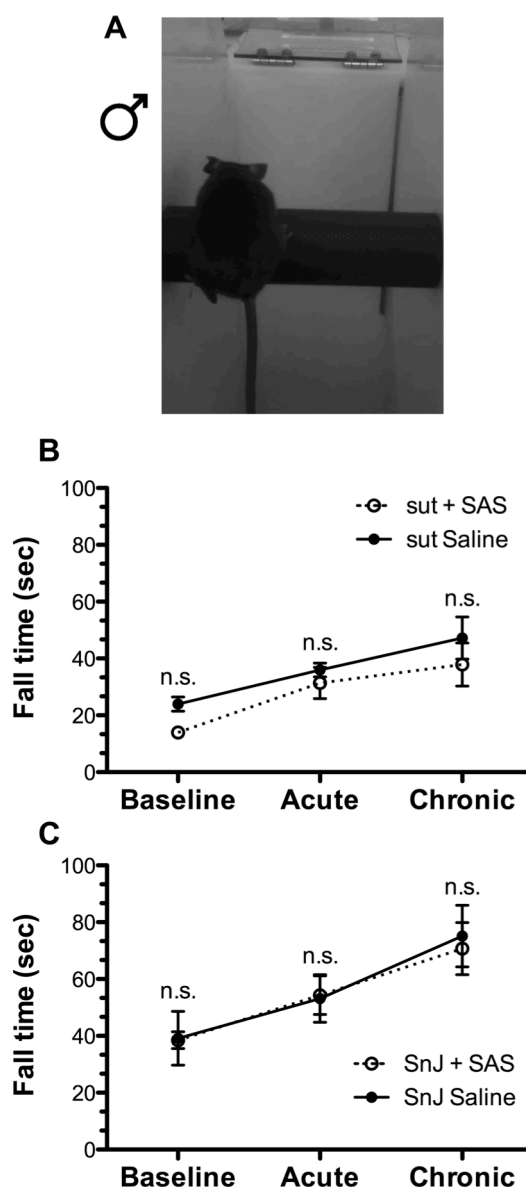
Figure 5 Male sut and SnJ Mice During the Rotarod Task with Sulfasalazine or Saline Treatment

 A, image of a mouse on the rotarod B, C, length of time before the mouse falls during the rotarod task for male sut mice (A) or SnJ mice (B) with SAS treatment or saline over three time periods, baseline, before any injections, acute, after the first injection or chronic, after twice daily injections for seven days.(n=7-8 for each treatment/genotype, animal has five trials per time period).
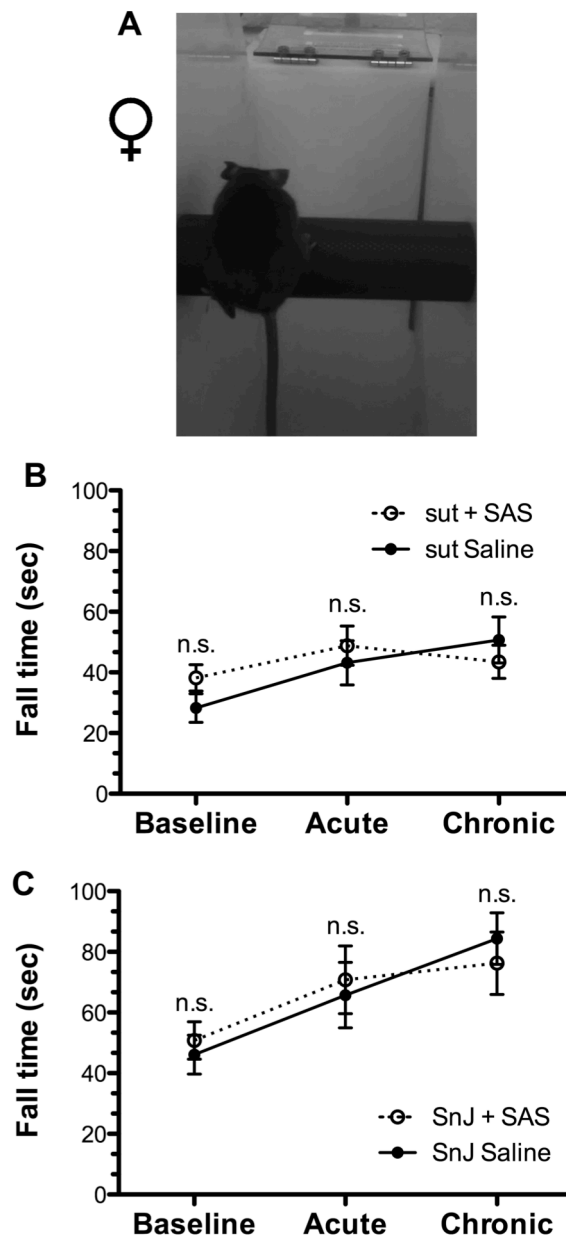
Figure 6 Female sut and SnJ Mice During the Rotarod Task with Sulfasalazine or Saline Treatment

A, image of a mouse on the rotarod B, C, length of time before the mouse falls during the rotarod task for female sut mice (A) or SnJ mice (B) with SAS treatment or saline over three time periods, baseline, before any injections, acute, after the first injection or chronic, after twice daily injections for seven days. (n=7-8 for each treatment/genotype, animal has five trials per time period).
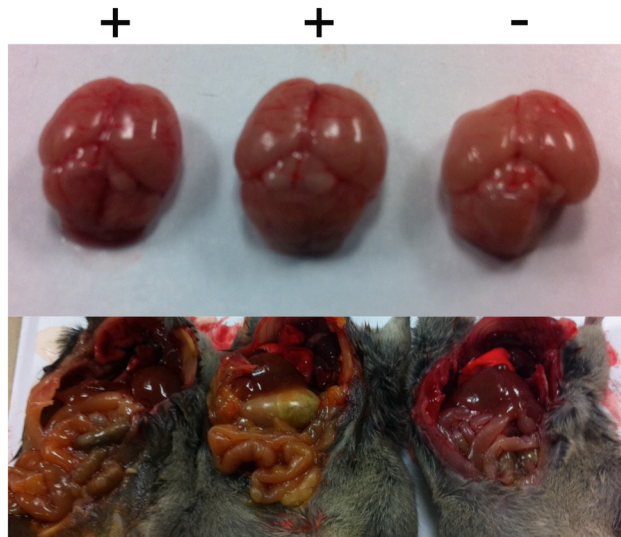
Figure 7 Injection of Sulfasalazine Changes the Color of the Gut but not the Brain.

The two brains and guts on the left were given SAS, but the one on the right was not. You can see the yellow color of the SAS in the + animals, but not in the – animal.

4.3 Discussion

In summary, neither males nor females of *sut* or *SnJ* genotype injected with sulfasalazine (SAS) had impairment in the four-arm spontaneous alternation task or in the rotarod task. Therefore, either the reduction in number of arm choices seen in *sut* animals is not due to system xc- activity, or perhaps SAS is not permeable to the blood brain barrier.

To minimize disruption to the brain, the injection of SAS was given intraperitoneal (I.P.) similar to the study by Chung et al., 2005. However Chung et al was giving SAS to animals that already potentially had a disrupted blood-brain-barrier since they had induced gliomas (Chung et al., 2005). The SAS had a very distinct color to it, and therefore I tried to determine if it had penetrated into the brains of animals by comparing the yellow coloration of the guts of animals given SAS treatment to their brains to see if the same yellow coloration was present in the brain as well (Figure 7). It was impossible to tell if the SAS had permeated into the brain using this method, however we could detect its presence in the gut.

Perhaps pharmacologically manipulating system xc- through SAS treatment was ineffective in phenocopying *sut* behavior because it did not cross the blood brain barrier. It is still possible that the *sut* behavior was not due to loss of system xc- but through some background effect of the mutation in that background. One way to determine if SAS was ineffective or if the behavior is

not due to system xc- is to look at multiple alleles of the same mutation in several different backgrounds.

5   COMPARISON OF BEHAVIOR FOR THREE ALLELES OF XCT MUTANTS

5.1 Introduction

It is still unclear whether the reduction in number of arm choices seen in *sut* animals is due to loss of system xc- since we cannot tell if SAS crosses the blood brain barrier and act as a system xc- inhibitor. Another way to test if the behavior seen in *sut* mice is through system xc- is to look at the behavior across multiple alleles and backgrounds. Fortunately, Sato et al cloned the *Slc7a11* gene, and inserted a GFP-tagged stop codon in the 1st exon in a C57/Bl6 background strain (hereafter called *xCT* and *B6*) (Sato et al., 2005). We can now attempt to ascertain if the deficits seen in *sut* mice are due to system xc- activity or are a product of the background strain by testing both *sut* and *xCT* animals in the same behavioral tests. In addition, we can attempt to control for background by crossing the two knockout strains and their controls (Hereafter called *xCT/sut* and *B6/SnJ*).

Both males and females of both genotypes were tested in the rotarod task, four-arm spontaneous alternation task, open-field maze, three-arm spontaneous alternation task, and delayed spontaneous alternation task and were compared to their controls. Any behaviors that could be attributable to loss of system xc- must occur in both mutants and the cross; otherwise it could be an effect of the combination of the mutation with the background strain.

5.2 Results

5.2.1 System xc- knockout mice performance on the rotarod task

To show that system xc- knockouts do not show any motor deficits both male and female *sut* and *xCT* mice were tested on a rotarod over six days. As previously shown, male *sut* mice did not have impairment in the rotarod task over six days compared to their controls (Two-way ANOVA with matching fall time in seconds for males day 1, *sut* 54.2 ± 4.7 seconds n=10, *SnJ* 56.7 ± 6.8 seconds, n=10 p>0.05; day 2, *sut* 61.3 ± 6.2 seconds, n=10, *SnJ* 77.0 ± 8.8 seconds, n=10 p>0.05; day 3, *sut* 66.2 ± 10.4 seconds, n=10, *SnJ* 92.4 ± 7.4 seconds, n=10 p>0.05; day 4 *sut* 91.5 ± 9.3 seconds, n=10, *SnJ* 97.3 ± 7.4 seconds, n=10 p>0.05; day 5, *sut* 115.3 ± 11.3 seconds, n=10, *SnJ* 117.7 ± 9.9 seconds, n=10 p>0.05; day 6, *sut* 111.7 ± 11.1 seconds, n=10, *SnJ* 115.9 ± 8.2 seconds, n=10 p>0.05, Figure 1B).

Male *xCT* mice also did not have impairment in the rotarod task over six days compared to their controls (Two-way ANOVA with matching fall time in seconds for males day 1, *xCT* 101.9 ± 5.0 seconds n=10, *B6* 77.2 ± 7.5 seconds, n=10 p>0.05; day 2, *xCT* 144.0 ± 15.7 seconds, n=10, *B6* 112.3 ± 11.1 seconds, n=10 p>0.05; day 3, *xCT* 130.7 ± 10.3 seconds, n=10, *B6* 147.3 ± 8.1 seconds, n=10 p>0.05; day 4 *xCT* 161.4 ± 10.0 seconds, n=10, *B6* 144.2 ± 12.5 seconds, n=10 p>0.05; day 5, *xCT* 143.1 ± 14.7 seconds, n=10, *B6* 180.4 ± 8.8 seconds, n=10 p>0.05; day 6, *xCT* 184.0 ± 22.0 seconds, n=10, *B6* 168.4 ± 13.9 seconds,

n=10 p>0.05, Figure 1C). Thus, neither *sut* nor *xCT* males were impaired in the

rotarod task over six days compared to their controls.
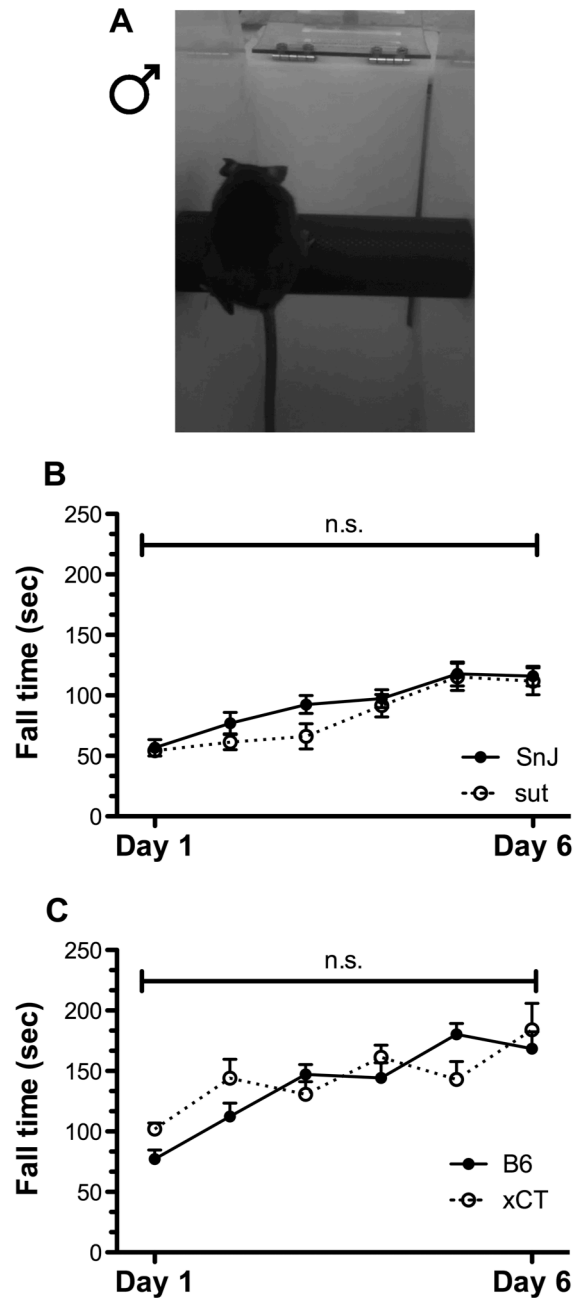
Figure 1 Male System xc- Mutants Performance on a Rotarod Task Over Six Days.

A, picture of a mouse on the rotarod B, sut and SnJ male performance over 6 days on the rotarod task. C, B6 and xCT male performance on the rotarod task over 6 days (n=10 for each genotype).

As shown previously *sut* females were not impaired in the rotarod task over six days compared to their controls (Two-way ANOVA with matching fall time in seconds for females day 1, *sut* 84.5 ± 6.8 seconds n=10, *SnJ* 75.1 ± 4.7 seconds, n=10 p>0.05; day 2, *sut* 76.3 ± 8.7 seconds, n=10, *SnJ* 113.7 ± 6.5 seconds, n=10 p>0.05; day 3, *sut* 97.5 ± 12.9 seconds, n=10, *SnJ* 132.6 ± 10.0 seconds, n=10 p>0.05; day 4 *sut* 129.7 ± 15.7 seconds, n=10, *SnJ* 150.0 ± 14.9 seconds, n=10 p>0.05; day 5, *sut* 140.2 ± 15.7 seconds, n=10, *SnJ* 177.5 ± 14.8 seconds, n=10 p>0.05; day 6, *sut* 150.2 ± 16.8 seconds, n=10, *SnJ* 192.3 ± 11.8 seconds, n=10 p>0.05, Figure 2B).

Female *xCT* mice were also not impaired in the six-day rotarod task compared to their controls (Two-way ANOVA with matching fall time in seconds for females day 1, *xCT* 108.6 ± 12.9 seconds n=10, *B6* 100.8 ± 9.6 seconds, n=10 p>0.05; day 2, *xCT* 150.7 ± 13.7 seconds, n=10, *B6* 149.6 ± 13.8 seconds, n=10 p>0.05; day 3, *xCT* 164.5 ± 13.0 seconds, n=10, *B6* 174.2 ± 14.3 seconds, n=10 p>0.05; day 4 *xCT* 185.9 ± 14.1 seconds, n=10, *B6* 194.5 ± 10.9 seconds, n=10 p>0.05; day 5, *xCT* 207.4 ± 11.5 seconds, n=10, *B6* 203.1 ± 11.0 seconds, n=10 p>0.05; day 6, *xCT* 206.5 ± 17.1 seconds, n=10, *B6* 218.3 ± 13.5 seconds, n=10 p>0.05, Figure 2C). Thus neither *sut* nor *xCT* females are impaired in the six-day rotarod task as compared to their controls.
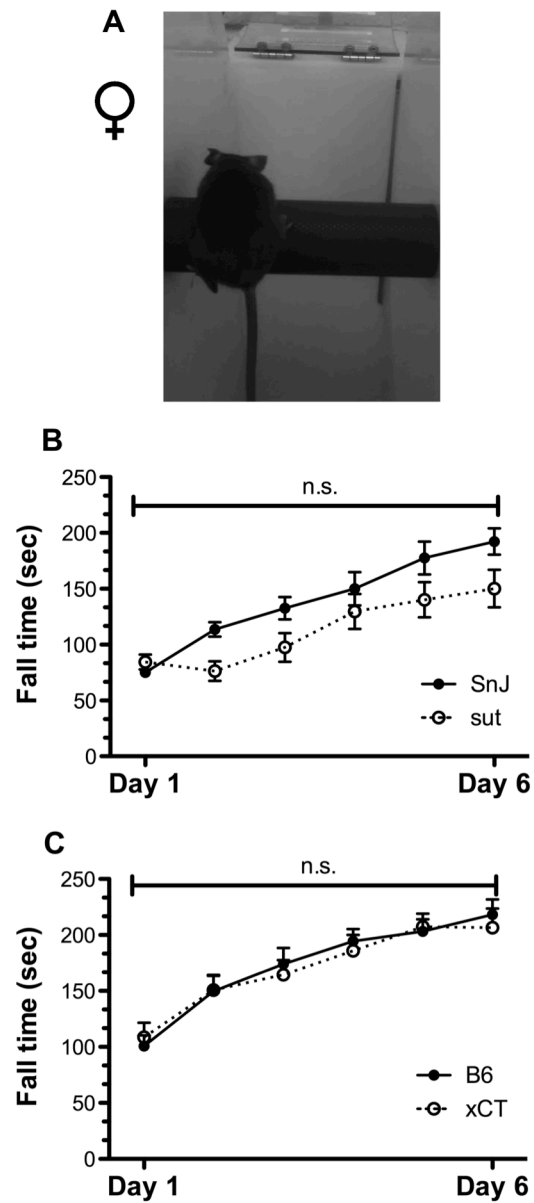
Figure 2 Female System xc- Mutants Performance on a Rotarod Task Over Six Days.

A, picture of a mouse on the rotarod B, sut and SnJ female performance over 6 days on the rotarod task. C, B6 and xCT male performance on the rotarod task over 6 days (n=10 for each genotype).

5.2.2 System xc- mice performance in a four-arm spontaneous alternation maze

xCT knockout mice were tested in the four-arm spontaneous alternation task to test if they had similar impairments to the *sut* genotype. To control for background *xCT/sut* mice and their background were also tested. As shown previously male *sut* mice were not impaired in percent alternations (Two-tailed t-test of spontaneous alternation percent, *sut* 18.5 ± 4.9%, n=10, *SnJ* 20.6 ± 3.9% n=10, p=0.74, Figure 3B). Male *xCT* mice were also not impaired in the percent alternations compared to their controls (Two-tailed t-test of spontaneous alternation percent, *xCT* 27.5 ± 2.9% n=14, *B6* 31.8 ± 3.0%, n=11, p=0.32, Figure 3B). Males of the *xCT/sut* cross also had no deficits in this task compared to controls (Two-tailed t-test of spontaneous alternation percent, *xCT/sut* 28.4 ± 3.2% n=10, *B6/SnJ* 32.8 ± 2.6% n=10, p=0.31, Figure 3B).   None of the male system xc- mutants had any impairment in percent spontaneous alternation in the four-arm maze compared to their controls.

As shown previously, *sut* males did have a reduction in their number of arm choices in the maze compared to their controls (Two-tailed t-test of number of arm choices, *sut* 19.9 ± 3.9 choices n=10, *SnJ* 32.7 ± 4.3 choices, n=10, p=0.04 Figure 3C). However, male *xCT* mice did not have a reduced number of arm choices in this task compared to their controls (Two-tailed t-test of number of arm choices, *xCT* 28.7 ± 1.3 choices, n=14, *B6* 32.6 ± 2.1 choices n=11, p=0.12 Figure 3C). The *xCT/sut* cross males also had a reduction in the number of arm choices similar to the *sut* mice as compared to their controls (Two-tailed t-test of

number of arm choices, *xCT/sut* 27.8 ± 1.8 choices n=10, *B6/SnJ* 34.0 ± 1.7

choices n=10, p=0.02 Figure 3C). Both *sut* and *xCT/sut* have a reduction in

number of arm choices in the four-arm spontaneous alternation task compared to

their controls, however the *xCT* strain does not share this impairment.

As shown previously female *sut* mice were not impaired in the alternation

percent (Two-tailed t-test of spontaneous alternation percent, *sut*, 12.2 ± 1.9%

n=69, *SnJ* 17.1 ± 1.9% n=56, p=0.07, Figure 4B). Female *xCT* mice were also

not impaired in the percent alternations compared to their controls (Two-tailed t-

test of spontaneous alternation percent, *xCT* 28.3 ± 1.3% n=39, *B6* 27.6 ± 1.8%

n=39, p=0.76, Figure 4B). Females of the *xCT/sut* cross also had no deficits in

this task compared to controls (Two-tailed t-test of spontaneous alternation

percent, *xCT/sut* 37.8 ± 4.1% n=11, *B6/SnJ* 38.4 ± 3.8% n=10 p=0.92, Figure

4B).   Thus, none of the female system xc- mutants had any impairment in

spontaneous alternation percent in the four-arm maze compared to their controls.

As shown previously, *sut* females did have a reduction in their number of

arm choices in the maze compared to their controls (Two-tailed t-test of number

of arm choices, *sut* 13.0 ± 1.5 choices n=68, *SnJ* 19.8 ± 1.8 choices n=57,

p=0.004 Figure 4C). However, female *xCT* mice did not have a reduced number

of arm choices in this task compared to their controls (Two-tailed t-test of number

of arm choices, *xCT* 29.5 ± 1.0 choices n=39, *B6* 30.7 ± 1.1 choices n=39,

p=0.43 Figure 4C). The *xCT/sut* cross females also did not have a reduction in

the number of arm choices similar to the *xCT* mice as compared to their controls

(Two-tailed t-test of number of arm choices, *xCT/sut* 28.0 ± 1.7 n=11, *B6/SnJ*

28.4 ± 2.0 n=10, p=0.88 Figure 4C). Only *sut* females show a reduction in

number of arm choices compared to their controls. Both *xCT* and the *xCT*/*sut*

cross do not make fewer arm choices compared to their controls.

Figure 3 Male xCT Mutants Performance in a 4 arm Spontaneous Alternation Maze

A, diagram of the layout of the maze. B, % alternation ability for male xCT mutants (n=10 for each genotype). C number of arm choices for male xCT mutants (*=p<0.05, n=10 for each genotype)

Figure 4 Female xCT Mutants Performance in a 4 arm Spontaneous Alternation Maze

A, diagram of the layout of the maze. B, % alternation ability for female xCT mutants (n=10 for each genotype). C number of arm choices for male xCT mutants (**=p<0.01, n=10 for each genotype).

5.2.3 System xc- performance in an open field maze

To test if the *sut* arm choice phenotype is due to motor and anxiety effects, and to see if system xc- might be related to motor and anxiety behavior, all three genotypes were tested in an open-field maze. Male *sut* males did explore the maze less than their controls as indicated by fewer grid crossings (Two-tailed t-test of number of grid crossings, *sut* 64.9 ± 11.6 crossings n=14, *SnJ* 108.5 ± 10.5 crossings n=11, p=0.01 Figure 5C). However, neither male *xCT* nor *xCT/sut* animals made fewer grid crossings compared to their controls (Two-tailed t-test of number of grid crossings, *xCT* 127.5 ± 11.6 crossings n=8, *B6* 123.3 ± 4.4 crossings n=11, p=0.71; *xCT/sut* 113.2 ± 6.7 crossings n=10, *B6/SnJ* 91.9 ± 8.7 crossings n=10, p=0.07 Figure 5C). These results indicate that only *sut* males made fewer grid crossings than their controls; the other two genotypes did not have the same behavioral phenotype and did not have a reduction in grid crossings.

To examine if system xc- mutants also have an anxiety-related phenotype, percent time in the middle of the open-field maze was quantified for each genotype. Males of all three genotypes did not spend significantly different time in the middle of the maze than their controls (Two-tailed t-test of percent time in the middle of the maze, *sut*, 11.3 ± 2.5% n=14, *SnJ* 9.3 ± 1.2% n=11 p=0.52, *xCT* 11.7 ± 1.0% n=8, *B6* 10.5 ± 0.83% n=11, p=0.35; *xCT/sut* 10.7 ± 1.1% n=10, *B6/SnJ* 9.4 ± 1.4% n=10, p=0.47 Figure 5D). Thus, system xc- males do not have an anxiety-related behavioral phenotype as tested by time in the middle of an open field.

Figure 5 Male xCT Mutants Performance in the Open Field Maze

A, an example of the layout of the maze. B an example of how "middle of the maze" was quantified C, male xCT mutant number of grid crossings in the open field maze (n=10 for each genotype, * is significant at p<0.05). D, amount of time spent in the middle (n=10 per genotype).
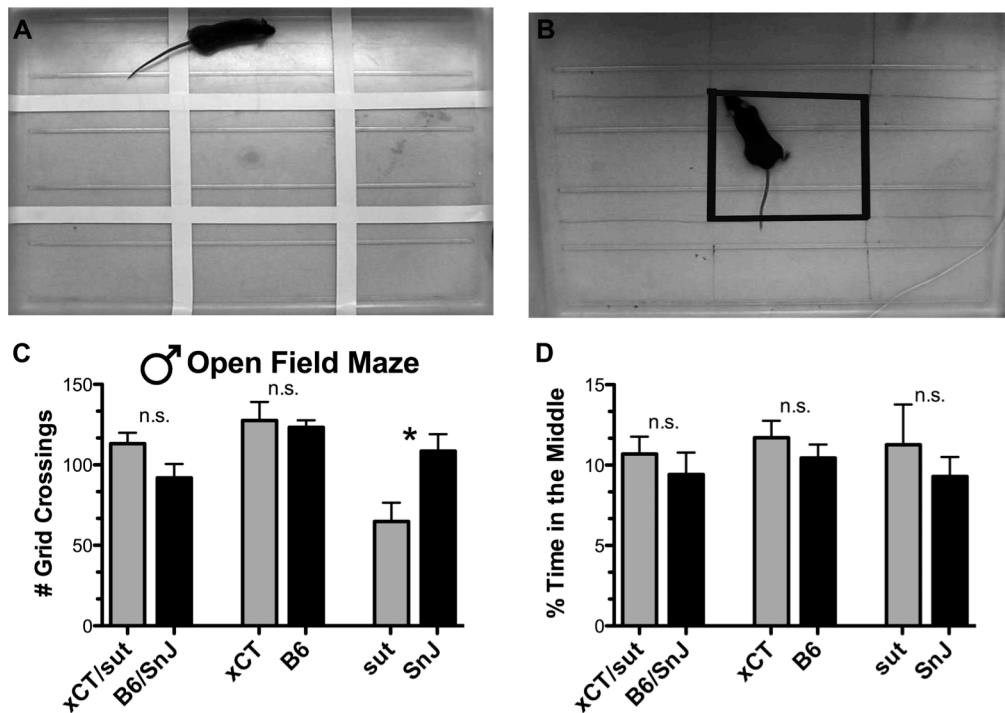
Female system xc- mice were also tested in the open-field task. None of the three genotypes made fewer grid crossings compared to their controls (Two-tailed t-test of number of grid crossings, *sut* 74.8 ± 10.3 crossings n=10, *SnJ* 68.7 ± 12.5 crossings n=10, p=0.71, *xCT* 95.3 ± 4.2 crossings n=10, *B6* 115.4 ± 8.1 crossings n=12, p=0.06; *xCT/sut* 117.5 ± 6.5 crossings n=11, *B6/SnJ* 99.5 ± 6.8 crossings n=10, p=0.07 Figure 6C). Females from all three system xc- mutants did not spend a greater percentage of time in the middle than their controls (Two-tailed t-test of percent time in the middle of the maze, *sut*, 6.6 ± 1.3% n=10, *SnJ* 7.2 ± 1.0% n=10 p=0.71, *xCT* 7.7 ± 1.1% n=10, *B6* 10.0 ± 1.1% n=12, p=0.15; *xCT/sut* 9.8 ± 1.1% n=11, *B6/SnJ* 9.1 ± 0.51% n=10, p=0.60 Figure 6D). These results indicate that female system xc- mice did not have any impairment in the open-field maze.
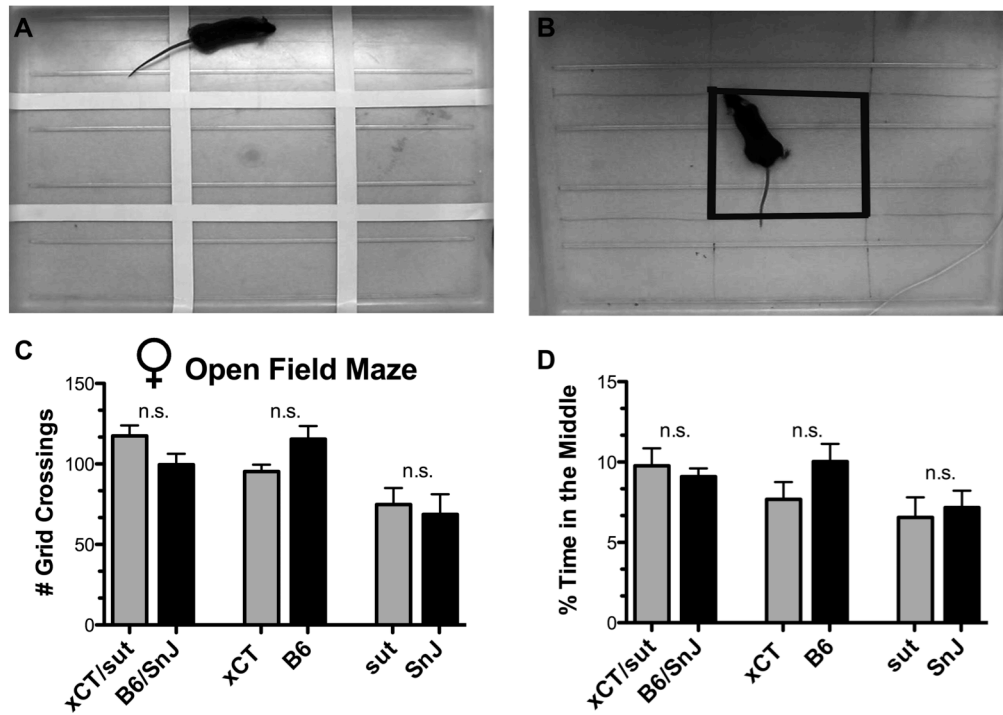
Figure 6 Female xCT Mutants Performance in the Open Field Maze

A, an example of the layout of the maze. B an example of how "middle of the maze" was quantified C, female xCT mutant number of grid crossings in the open field maze (n=10 for each genotype). D, amount of time spent in the middle (n=10 per genotype).

5.2.4 System xc- mutants performance in a three-arm spontaneous alternation maze

A more traditional version of the spontaneous alternation task is the y-maze or three-arm alternation maze. This maze typically has more alternations and arm choices due to fewer possibilities, therefore I wanted to test system xc- mutants in this maze to get at more subtle differences in the mouse's spontaneous alternation ability (Lalonde, 2002; Crawley, 2008). In the four-arm version of this maze none of the mice had a deficit related to their ability to alternate in the maze (Figure 3, 4). However, *sut* males alternate less in the three-arm maze compared to their controls (Two-tailed t-test of spontaneous alternation percent, *sut* 20.2 ± 6.7% n=14, *SnJ* 49.3 ± 4.1% n=11, p=0.002 Figure 7B). In contrast, *xCT* and *xCT/sut* animals do not have a deficit in spontaneous alternation compared to their controls (Two-tailed t-test of spontaneous alternation percent, *xCT* 59.7 ± 2.9% n=10, *B6* 61.2 ± 3.8% n=11, p=0.75, *xCT/sut* 60.2 ± 5.6% n=10, *B6/SnJ* 59.1 ± 5.3% n=10, p=0.89 Figure 7B). Interestingly, in a three-arm maze *sut* males now show a deficit in alternation ability, however neither *xCT* nor *xCT/sut* animals have this deficit.

Consistent with the data from the four-arm maze, only *sut* males made fewer arm choices than their controls. *xCT* and *xCT/sut* animals were comparable to their controls (Two-tailed t-test of number of arm choices, *sut* 8.8

± 2.5 choices n=14, *SnJ* 22.6 ± 2.2 choices n=11, p=0.0006, *xCT* 22.7 ± 2.0

choices n=10, *B6* 22.4 ± 0.77 choices n=11, p=0.87, *xCT/sut* 22.8 ± 2.0 choices

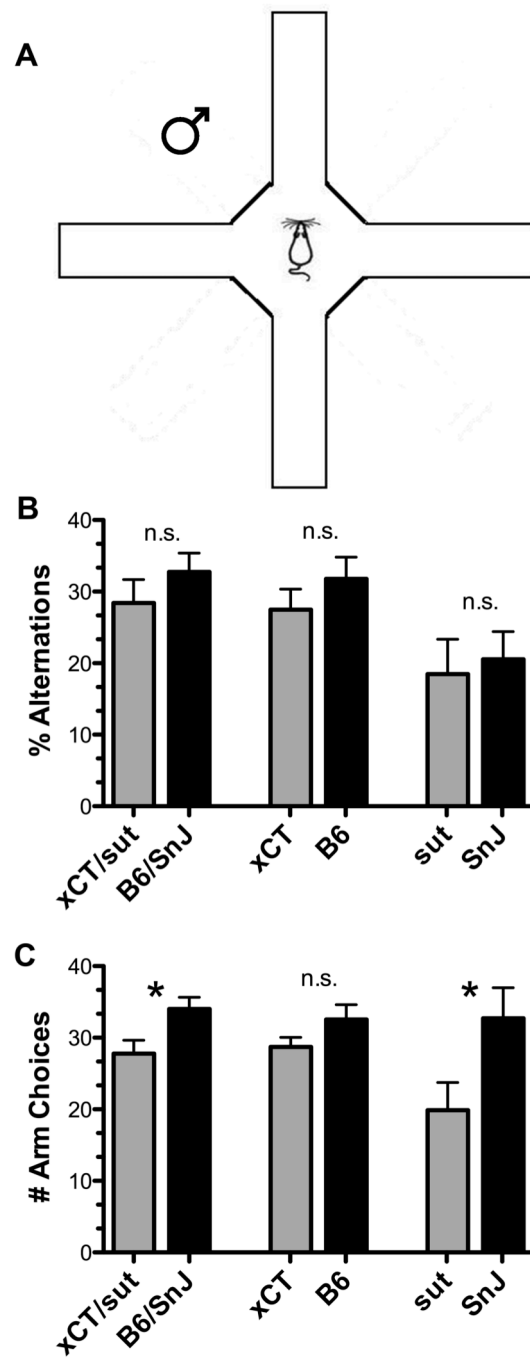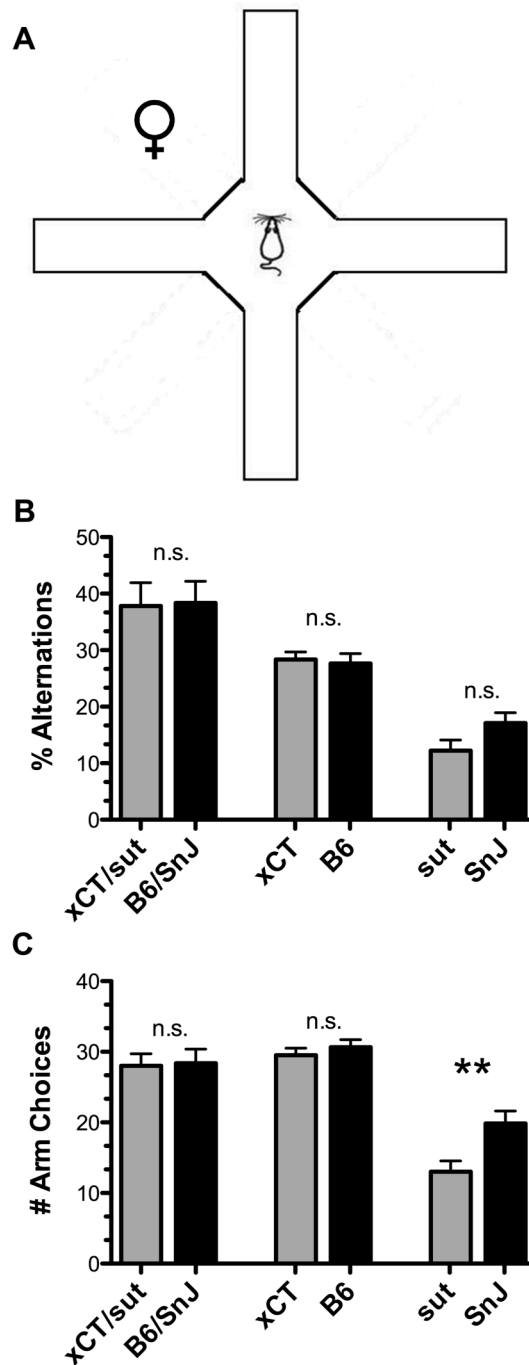n=10, *B6/SnJ* 22.8 ± 2.0 choices n=10, p=1.0 Figure 7C).
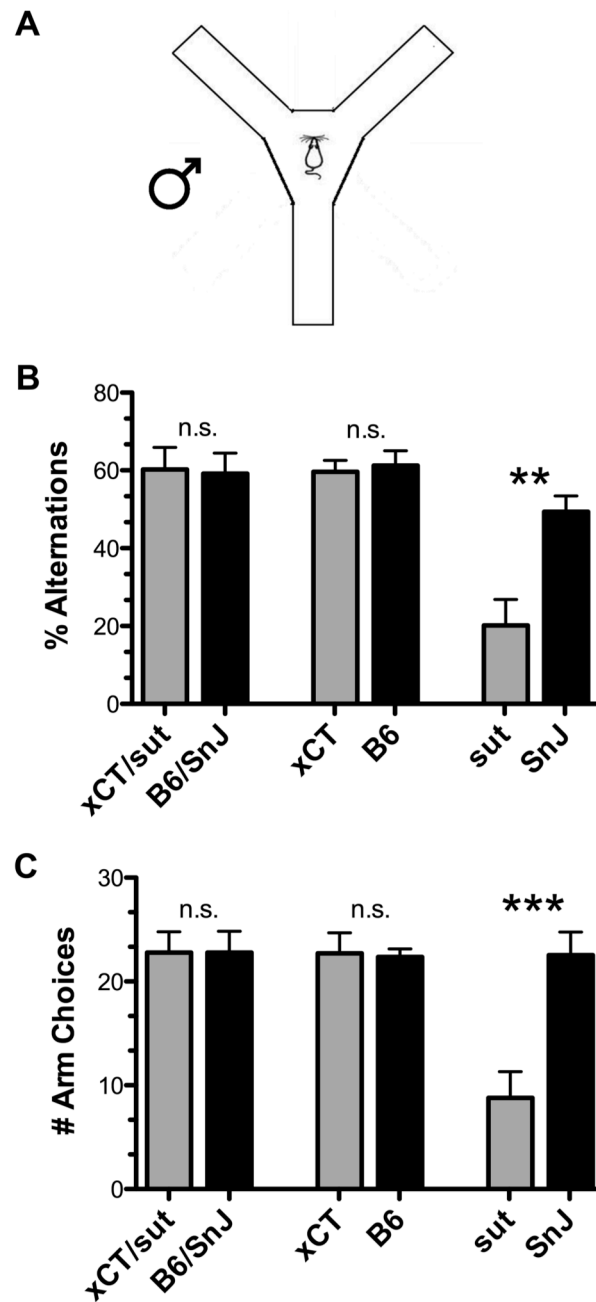
Figure 7 Male xCT Mutants Performance in a 3 arm Spontaneous Alternation Maze

A, diagram of the layout of the maze. B, % alternation ability for male xCT mutants (**=p<0.01, ***=p<0.001 n=10 for each genotype). C, number of arm choices for female xCT mutants (n=10 for each genotype)

Consistent with what was seen in males, female *sut* mice made fewer alternations than their controls, while *xCT* and *xCT/sut* animals were similar to their controls (Two-tailed t-test of spontaneous alternation percent, *sut* 26.9 ± 8.2% n=10, *SnJ* 54.9 ± 7.8% n=10, p=0.02, *xCT* 60.6 ± 4.5% n=10, *B6* 58.2 ± 3.4% n=12 p=0.66, *xCT/sut* 64.0 ± 3.8% n=11, *B6/SnJ* 64.8 ± 4.3% n=10 p=0.89, Figure 8B).   Unlike in the four-arm task, *sut* females did not show a significant reduction in the number of arm choices in the three-arm maze (Two-tailed t-test of number of arm choices, *sut* 13.7 ± 3.3 choices n=10 p=0.1, *SnJ* 20.9 ± 2.5 choices n=10, p=0.1 Figure 8C).   Consistent with males and data from the four-arm maze neither *xCT* nor *xCT/sut* animals showed a reduction in the number of arm choices in this task compared to their controls (Two-tailed t-test of number of arm choices, *xCT* 20.2 ± 1.6 choices n=10, *B6* 23.1 ± 1.6 choices n=12 p=0.21, *xCT/sut* 22.6 ± 1.4 choices n=11, *B6/SnJ* 19.6 ± 1.6 choices n=10 p=0.19, Figure 8C).   Unlike in the four-arm maze, female *sut* mice make fewer alternations, but not number of arm choices.   Consistent with the four-arm data, these data show that *xCT* and *xCT/sut* animals do not have any impairment in the three-arm spontaneous alternation task.
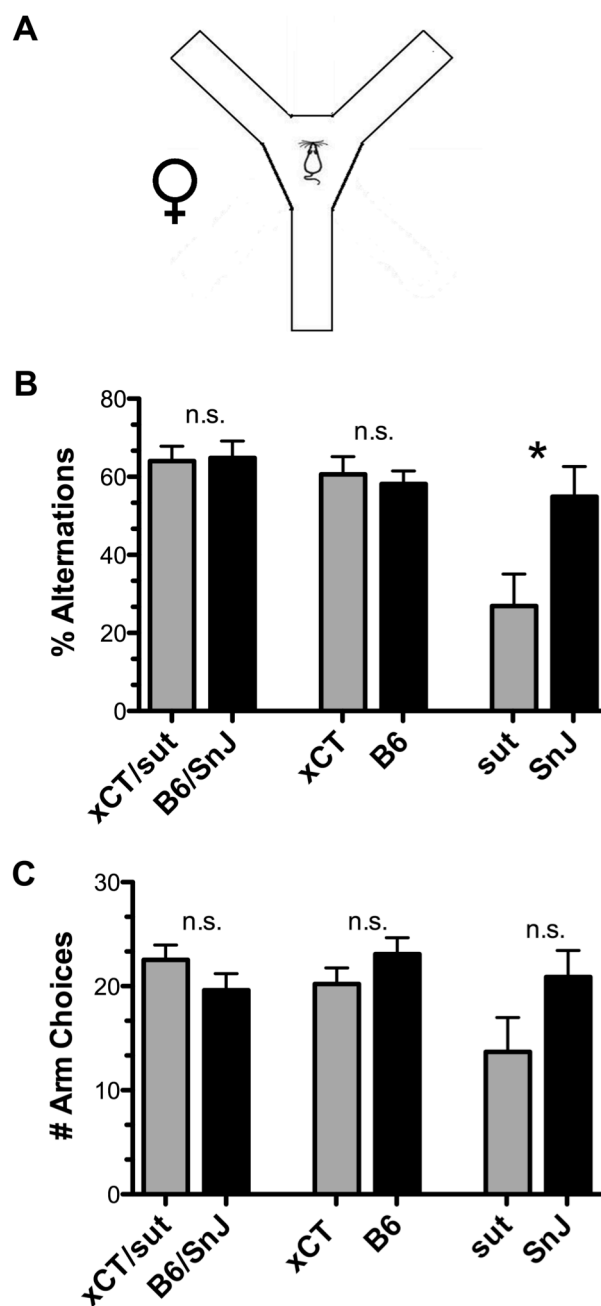
Figure 8 Female xCT Mutants Performance in a 3 arm Spontaneous Alternation Maze

A, diagram of the layout of the maze. B, % alternation ability for female xCT mutants (*=p<0.05, n=10 for each genotype). C, number of arm choices for female xCT mutants (n=10 for each genotype)

5.2.5 System xc- mutants performance in a delayed spontaneous alternation task

Another variation of the spontaneous alternation task is the delayed spontaneous alternation task. The benefit of this task is to remove the propensity of an animal from preferring right or left hand turns as well as it generally increases the number of arm choices since the animal should prefer to enter the novel arm (Lalonde, 2002). Male system xc- mice did not show any deficits in this task compared to their controls. Mutants from all three genotypes spent equivalent amounts of time in each arm compared to their controls (Two-way ANOVA without matching percent time in each arm, novel arm *sut* 38.6 ± 7.7% n=10, *SnJ* 32.8 ± 4.0% n=10 p>0.05, *xCT* 41.9 ± 7.2% n=10, *B6* 33.6 ± 3.8% n=10 p>0.05, *xCT/sut* 41.5 ± 5.4% n=10, *B6/SnJ* 42.4 ± 6.3% n=10 p>0.05; F1 *sut* 18.6 ± 5.7% n=10, *SnJ* 15.8 ± 3.1% n=10 p>0.05, *xCT* 19.6 ± 5.5% n=10, *B6* 23.4 ± 2.9% n=10 p>0.05, *xCT/sut* 34.2 ± 6.7% n=10, *B6/SnJ* 35.3 ± 7.3% n=10 p>0.05; F2 *sut* 20.9 ± 9.2% n=10, *SnJ* 17.9 ± 2.8% n=10 p>0.05, *xCT* 17.6 ± 3.0% n=10, *B6* 17.9 ± 2.2% n=10 p>0.05, *xCT/sut* 6.3 ± 0.9% n=10, *B6/SnJ* 7.9 ± 1.2% n=10 p>0.05, Figure 9B, C, D).

Males of all three genotypes generally did not make fewer arm choices than their controls, however *sut* males did choose arm F2 less frequently than their controls (Two-way ANOVA without matching number of arm choices, novel arm *sut* 3.2 ± 0.7 choices n=10, *SnJ* 4.8 ± 0.6 choices n=10 p>0.05, *xCT* 3.9 ± 0.6 choices n=10, *B6* 4.2 ± 0.8 choices n=10 p>0.05, *xCT/sut* 3.5 ± 0.6 choices n=10, *B6/SnJ* 3.8 ± 0.4 choices n=10 p>0.05; F1 *sut* 2.6 ± 0.8 choices n=10, *SnJ*

3.2 ± 0.7 choices n=10 p>0.05, *xCT* 2.2 ± 0.3 choices n=10, *B6* 3.2 ± 0.3 choices

n=10 p>0.05, *xCT/sut* 1.9 ± 0.4 choices n=10, *B6/SnJ* 2.9 ± 0.4 choices n=10

p>0.05; F2 *sut* 2.2 ± 0.4 choices n=10, *SnJ* 4.3 ± 0.6 choices n=10 p<0.05, *xCT*

3.7 ± 0.5 choices n=10, *B6* 3.8 ± 0.5 choices n=10 p>0.05, *xCT/sut* 2.3 ± 0.3

choices n=10, *B6/SnJ* 2.5 ± 0.3 choices n=10 p>0.05, Figure 10 B, C, D). Similar

to other mazes, *sut* males did make fewer arm choices than controls in one of the

arms, while the other two genotypes behaved similarly to their controls.
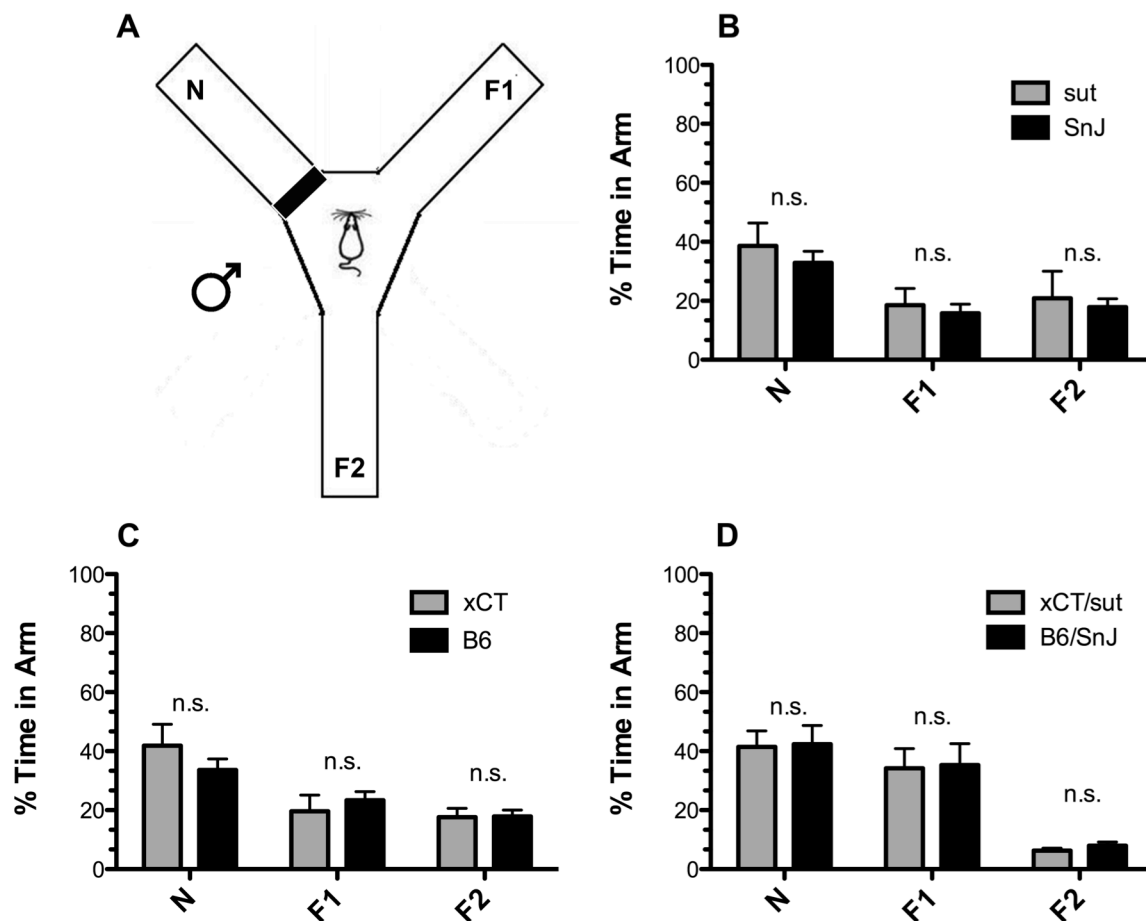
Figure 9 Male xCT Mutants % Time in Each Arm in the Delayed Spontaneous Alternation Maze

A, diagram of the maze B, C, D, percent time spent in each arm for sut and SnJ (B), xCT and B6 (C), and xCT/sut and B6/SnJ (D) N=novel arm, F1 and F2 are the two arms the animals were previously exposed to (n=10 for each genotype).
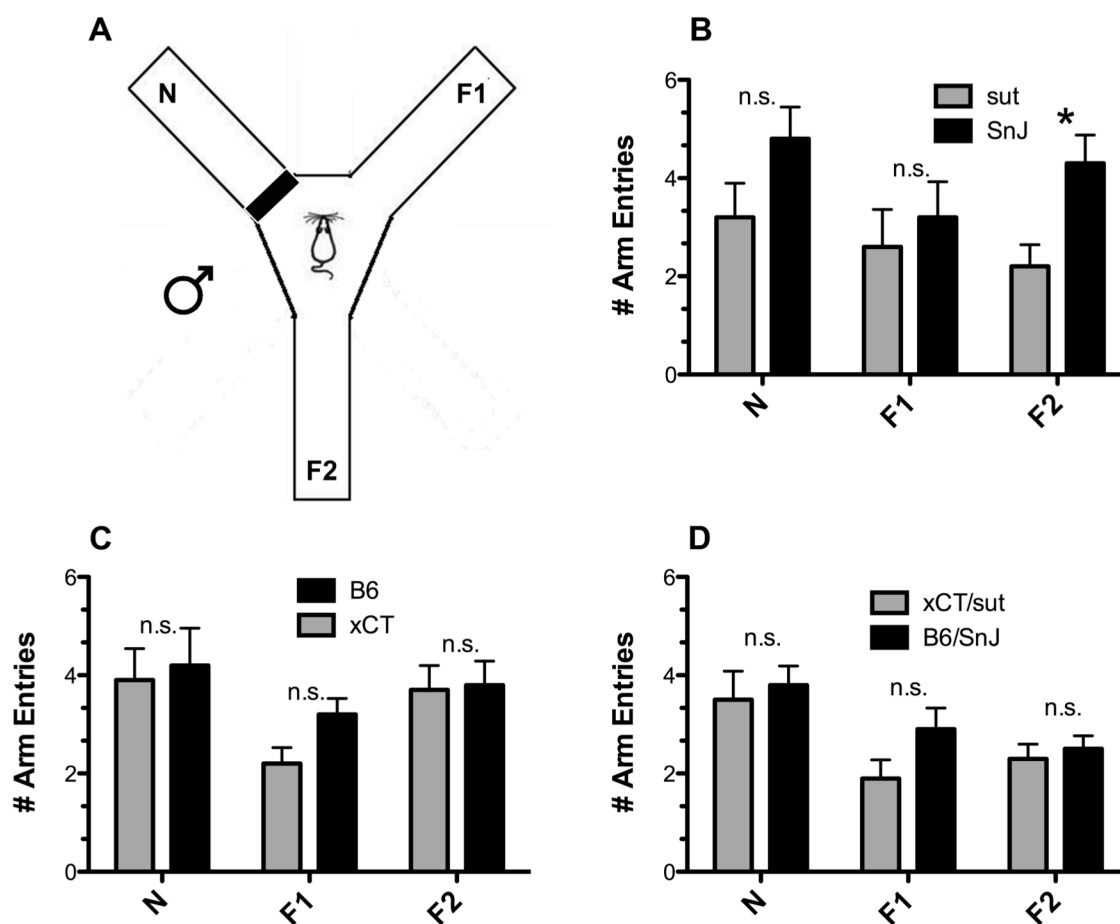
Figure 10 Male xCT Mutants # Arm Choices in the Delayed Spontaneous Alternation Maze

A, diagram of the maze B, C, D, number of arm choices for sut and SnJ (B), xCT and B6 (C), and xCT/sut and B6/SnJ (D) N=novel arm, F1 and F2 are the two arms the animals were previously exposed to (n=10 for each genotype, *=$p < 0.05$).

Female system xc- mutants also did not spend significantly less time in the three different arms in this task than their controls (Two-way ANOVA without matching percent time in each arm, novel arm *sut* 33.7 ± 6.3% n=10, *SnJ* 31.0 ± 4.1% n=10 p>0.05, *xCT* 38.9 ± 8.1% n=10, *B6* 33.2 ± 5.8% n=10 p>0.05, *xCT/sut* 30.2 ± 7.3% n=10, *B6/SnJ* 45.2 ± 6.4% n=10 p>0.05; F1 *sut* 22.8 ± 5.7% n=10, *SnJ* 22.3 ± 3.8% n=10 p>0.05, *xCT* 12.4 ± 4.3% n=10, *B6* 12.5 ± 3.2% n=10 p>0.05, *xCT/sut* 36.6 ± 9.4% n=10, *B6/SnJ* 26.8 ± 7.8% n=10 p>0.05; F2 *sut* 23.6 ± 9.1% n=10, *SnJ* 18.4 ± 2.4% n=10 p>0.05, *xCT* 27.4 ± 7.4% n=10, *B6* 23.3 ± 5.4% n=10 p>0.05, *xCT/sut* 14.3 ± 4.6% n=10, *B6/SnJ* 13.2 ± 3.2% n=10 p>0.05, Figure 11 B, C, D).

Similar to males, female *sut* mice made fewer F2 arm choices than their controls while the other two genotypes behaved similarly to their controls (Two-way ANOVA without matching number of arm choices, novel arm *sut* 5.0 ± 0.9 choices n=10, *SnJ* 5.4 ± 0.4 choices n=10 p>0.05, *xCT* 3.7 ± 0.6 choices n=10, *B6* 3.6 ± 0.5 choices n=10 p>0.05, *xCT/sut* 3.2 ± 0.7 choices n=10, *B6/SnJ* 3.9 ± 0.5 choices n=10 p>0.05; F1 *sut* 3.0 ± 0.6 choices n=10, *SnJ* 4.6 ± 0.8 choices n=10 p>0.05, *xCT* 1.7 ± 0.4 choices n=10, *B6* 1.9 ± 0.4 choices n=10 p>0.05, *xCT/sut* 2.1 ± 0.3 choices n=10, *B6/SnJ* 2.3 ± 0.4 choices n=10 p>0.05; F2 *sut* 2.9 ± 0.6 choices n=10, *SnJ* 5.2 ± 0.7 choices n=10 p<0.01, *xCT* 3.2 ± 0.5 choices n=10, *B6* 3.4 ± 0.5 choices n=10 p>0.05, *xCT/sut* 2.3 ± 0.4 choices n=10, *B6/SnJ* 2.8 ± 0.4 choices n=10 p>0.05, Figure 12 B, C, D). Similar to results from other mazes and results in males, female *sut* mice made fewer arm

choices in one of the arms, but had no other deficits. The other two knockouts performed similarly to their controls.
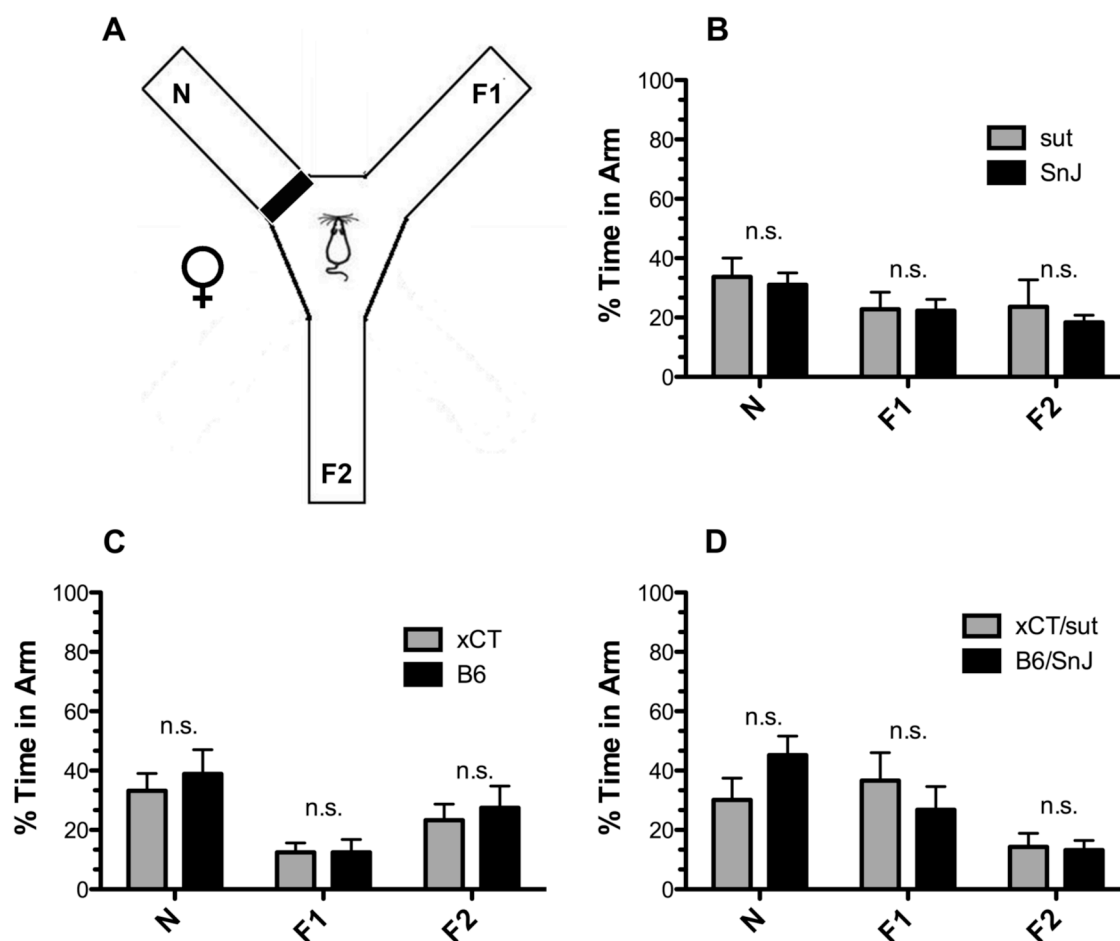
Figure 11 Female xCT Mutants % Time in Arm in the Delayed Spontaneous Alternation Maze

A, diagram of the maze B, C, D, number of arm choices for sut and SnJ (B), xCT and B6 (C), and xCT/sut and B6/SnJ (D) N=novel arm, F1 and F2 are the two arms the animals were previously exposed to (n=10 for each genotype).
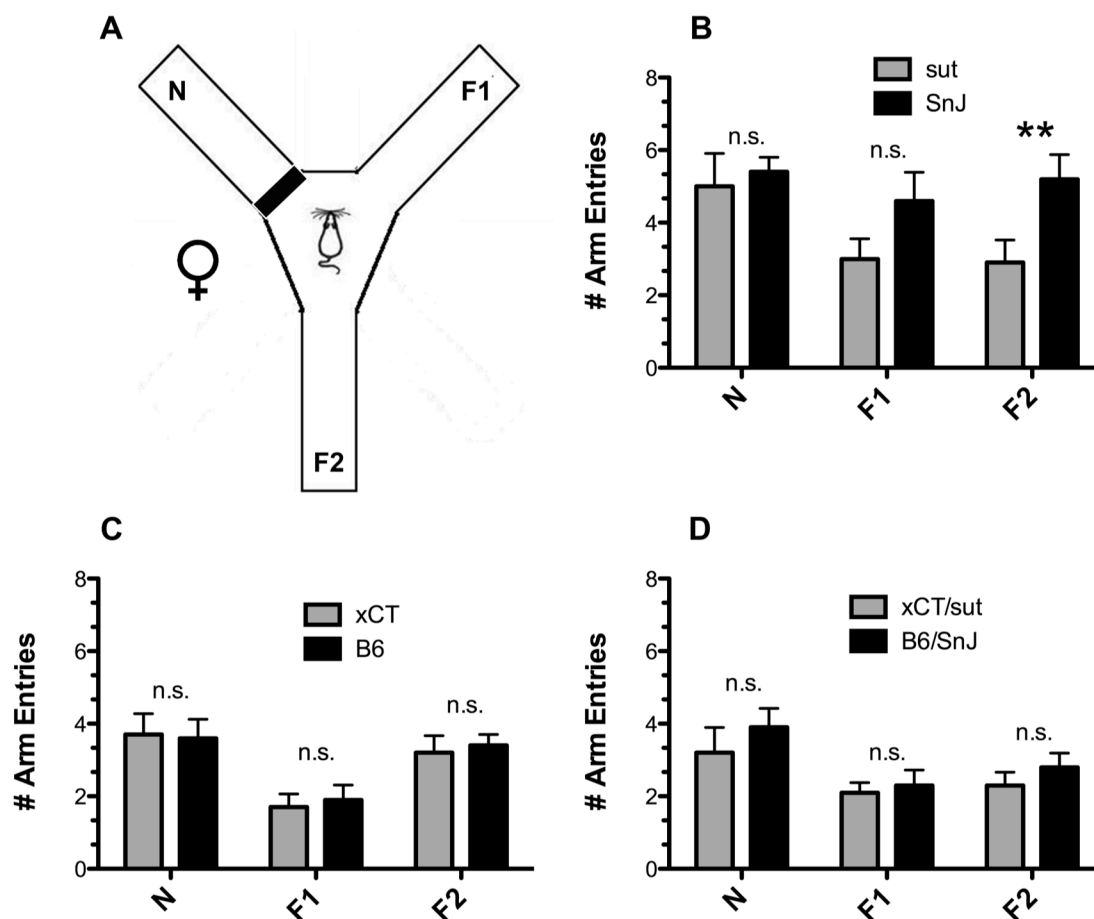
Figure 12 Female xCT Mutants # Arm Choices in the Delayed Spontaneous Alternation Maze

A, diagram of the maze B, C, D, number of arm choices for sut and SnJ (B), xCT and B6 (C), and xCT/sut and B6/SnJ (D) N=novel arm, F1 and F2 are the two arms the animals were previously exposed to (n=10 for each genotype, **=p<0.01).

5.3 Discussion

Male *sut* mutants had a similar phenotype in the three-arm spontaneous alternation maze as the four-arm maze shown previously in Chapter 3. However, in the three-arm maze *sut* mice also have a reduction in alternation ability. Males also exhibited a reduction in number of grid crossings in the open field maze compared to their controls. The reduced arm choices and spontaneous alternation ability shown by *sut* males could be explained by a motor deficit as shown by a reduction in number of grid crossings in the open field. This behavioral phenotype seems to be only exhibited by *sut* mice and not *xCT* mice and only the *xCT/sut* males in the four-arm version of the task. Therefore these behaviors cannot be attributed to system xc- loss.

Female *sut* mice also exhibit a reduction in arm choices in the four-arm spontaneous alternation maze, but do not show any open field deficits. Curiously, *sut* females do show a reduction in spontaneous alternation ability in the three-arm maze similar to males, but no reduction in arm choices. This suggests that perhaps male and female *sut* behavior may be different. However, similar to males, there were no behavioral deficits in female *xCT* or *xCT/sut* mice suggesting that the behaviors seen in *sut* mice are not due to loss of system xc-.

Studies have previously shown that *xCT* mice do have some behavioral deficits including a reduction in the number of arm choices in the delayed spontaneous alternation task and a reduced ability to spontaneously alternate (De Bundel et al., 2011). However, this study only found these deficits in young

mice (not old) and only in males. This suggests that whatever behavioral changes this transporter may be responsible for are probably very subtle in nature.

6 MICRODIALYSIS STUDIES OF GLUTAMATE LEVELS IN SUT AND XCT/SUT KNOCKOUTS

6.1 Introduction

One possible mechanism by which system xc- was proposed to have effects on behavior is through its extrusion of glutamate (Kalivas, 2009). Studies *in vivo* using microdialysis have shown that system xc- may account for around half of the extracellular glutamate in the striatum and hippocampus (Massie et al., 2011; De Bundel et al., 2011). Studies using pharmacological mechanisms have shown that blocking of system xc- using inhibitors also reduces glutamate levels by half (Baker et al., 2002). As yet, no one has measured whether there is a reduction in extracellular glutamate in the *sut* genotype. As shown, *sut* mice seem to be behaviorally different from the *xCT* genotype particularly in males and motor behavior (open-field maze). To further elucidate if *sut* animals as well as *xCT/sut* animals also have a reduction in glutamate, microdialysis measurements of glutamate levels were taken from the striatum and cerebellum in male *sut* mice and male and female *xCT/sut* mice and their controls. The striatum was chosen since it was previously shown to have reduced glutamate levels in *xCT* mice, and the cerebellum for the possible behavioral correlates with *sut* mice (Massie et al., 2011).

6.2 Results

6.2.1 Male *sut* glutamate levels in the striatum and the cerebellum

Male *sut* mice do not have a reduction in glutamate in the cerebellum compared to their controls (One-tailed Mann-Whitney glutamate levels cerebellum, *sut* 0.27 ± 0.06µM n=5, *SnJ* 0.81 ± 0.41µM n=6, p=0.33, Figure 1C). However, male *sut* mice did have a reduction of glutamate levels in the striatum compared to their controls (One-tailed Mann-Whitney glutamate levels striatum, *sut* 0.17 ± 0.12µM n=6, *SnJ* 0.35 ± 0.12µM n=6, p=0.047, Figure 1D). In summary, male *sut* mice do not have a reduction in glutamate levels in the cerebellum compared to their controls *SnJ,* but do have reduced glutamate levels in the striatum.
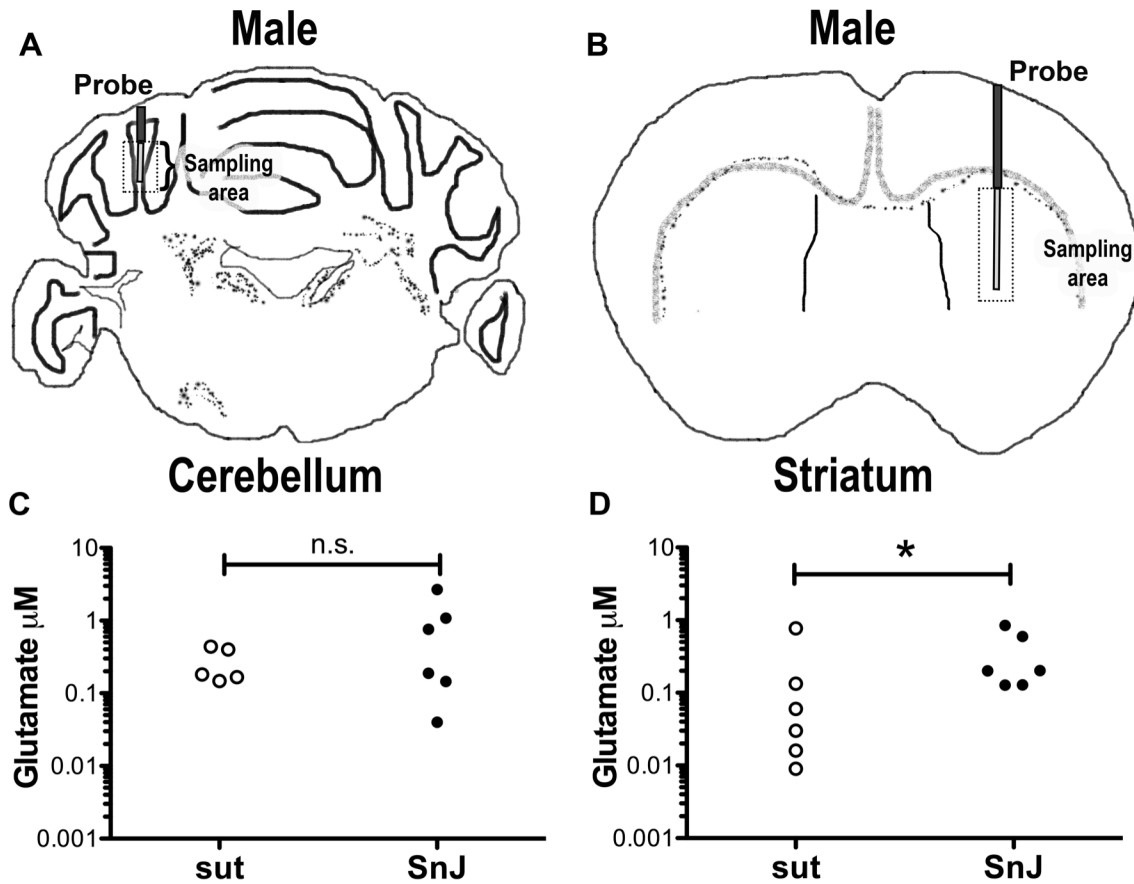
Figure 1 Male sut Mutant Glutamate Levels in the Cerebellum and Striatum.

A, an example of the probe placement in the cerebellum B an example of probe placement for the striatum C, male cerebellum glutamate levels for sut and SnJ (n=5 for sut, n=6 for SnJ). D, male striatum glutamate levels for sut and SnJ (n=5 for sut, n=6 for SnJ, *=significant at 0.05).

6.2.2 *xCT/sut* glutamate levels

Male *xCT/sut* animals did not have a reduction in glutamate levels in the striatum or cerebellum compared to their controls (One-tailed Mann-Whitney glutamate levels cerebellum, *xCT/sut* 0.50 ± 0.17μM n=6, *B6/SnJ* 0.55 ± 0.18μM n=6 p=0.44, Figure 2C; striatum, *xCT/sut* 0.32 ± 0.11μM n=6, *B6/SnJ* 0.39 ± 0.17μM n=6 p=0.50, Figure 2D). Female *xCT/sut* animals also did not have a reduction in glutamate levels compared to their controls in the striatum and cerebellum, though the levels in the cerebellum are very close to significant (p=0.053) (One-tailed Mann-Whitney glutamate levels cerebellum, *xCT/sut* 0.26 ± 0.078μM n=5, *B6/SnJ* 0.51 ± 0.11μM n=7 p=0.053, Figure 3C, striatum *xCT/sut* 0.41 ± 0.16μM n=6, *B6/SnJ* 0.56 ± 0.17μM n=7 p=0.31 Figure 3D). Thus, neither male nor female *xCT/sut* crosses have reduced glutamate levels in the striatum and cerebellum compared to their controls.

There was no significant difference in glutamate levels between males and females so the data was combined for a larger sample size (Two-way ANOVA with Bonferroni posttest glutamate levels in the striatum male *xCT/sut* 0.32 ± 0.11μM n=6; female *xCT/sut* 0.41 ± 0.16μM n=6 p>0.05. Glutamate levels in the striatum male *B6/SnJ*, 0.39 ± 0.17μM n=6; female *B6/SnJ* 0.56 ± 0.17μM n=7, p>0.05. Cerebellum glutamate levels male *xCT/sut* 0.50 ± 0.17μM n=5; female *xCT/sut* 0.26 ± 0.08μM n=5 p>0.05. Glutamate levels in the striatum male *B6/SnJ*, 0.55 ± 0.18μM n=6; female *B6/SnJ* 0.51 ± 0.11μM n=7, p>0.05).

Figure 2 Male xCT/sut Mutant Glutamate Levels in the Cerebellum and Striatum.

A, an example of the probe placement in tthe cerebellum B an example of probe placement for the striatum C, male cerebellum glutamate levels for xCT/sut and B6/SnJ (n=6 for xCT/sut, n=6 for B6/SnJ). D, male striatum glutamate levels for xCT/sut and B6/SnJ (n=6 for xCT/sut, n=6 for B6/SnJ). Images in A, B adapted from the Mouse Brain Library Atlases.
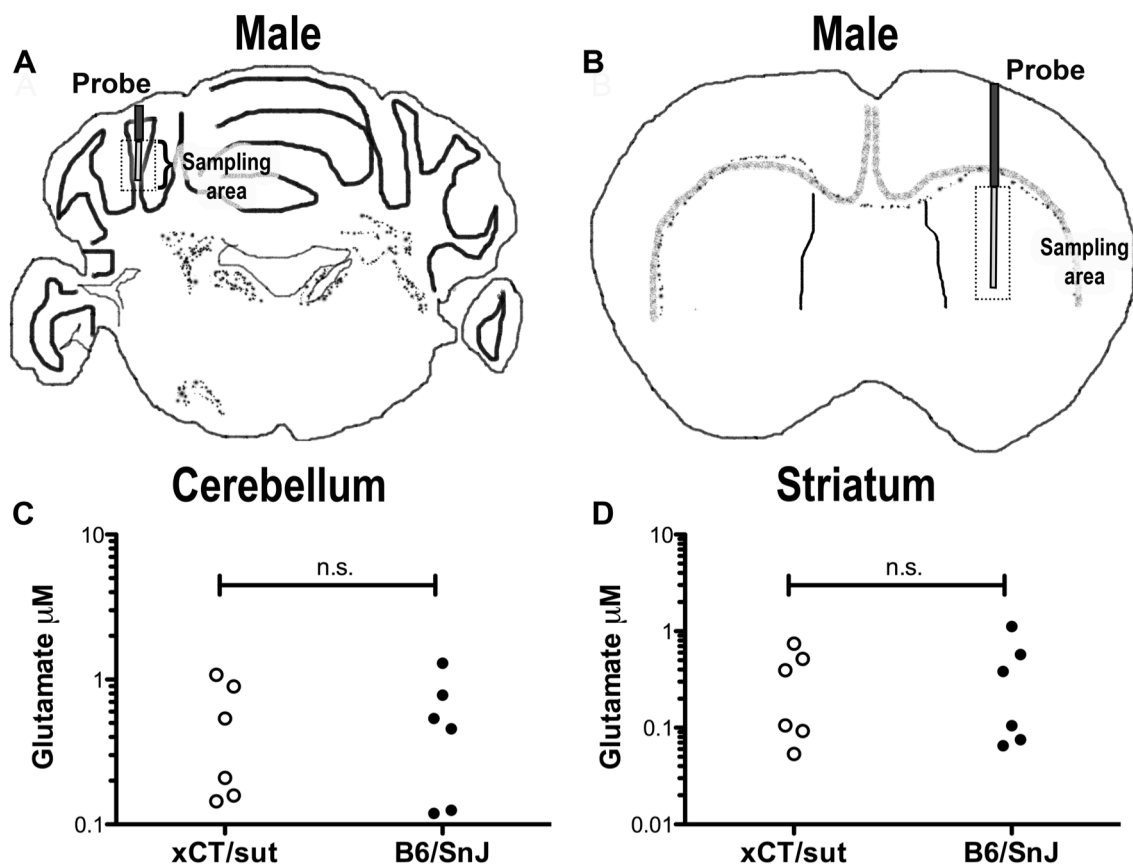
Figure 3 Female xCT/sut Mutant Glutamate Levels in the Cerebellum and Striatum

A, an example of the probe placement in tthe cerebellum B an example of probe placement for the striatum C, female cerebellum glutamate levels for xCT/sut and B6/SnJ (n=5 for xCT/sut, n=7 for B6/SnJ). D, female striatum glutamate levels for xCT/sut and B6/SnJ (n=6 for xCT/sut, n=7 for B6/SnJ).
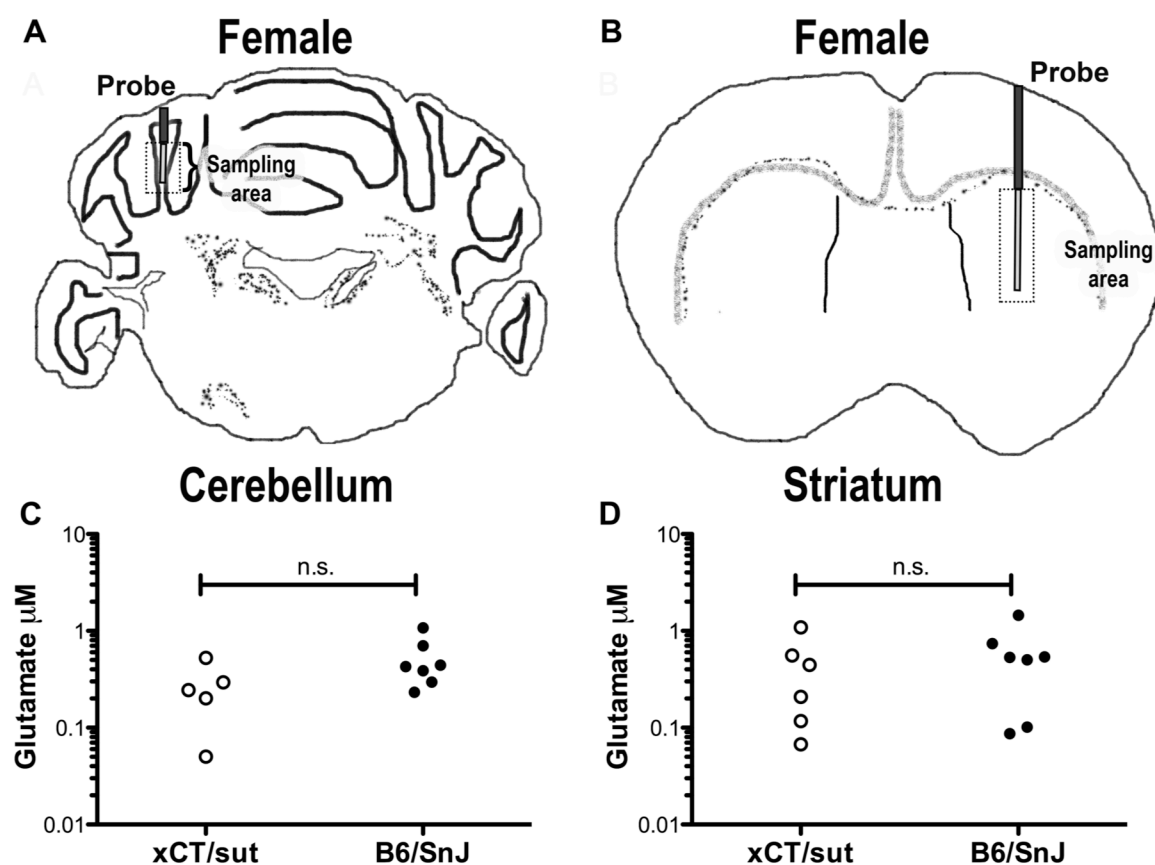
When comparing the combined data for males and females, there was no significant difference in glutamate levels between *xCT/sut* and their controls in the striatum or cerebellum (One-tailed Mann-Whitney glutamate levels in the striatum *xCT/sut* 0.37 ± 0.09µM n=12; *B6/SnJ* 0.48 ± 0.1µM n=13, p=0.32; cerebellum *xCT/sut* 0.39 ± 0.1µM n=11; *B6/SnJ* 0.53 ± 0.1µM n=13, p=0.16 Figure 4A and B).

6.2.3 Slow-flow glutamate collection for *xCT/sut* animals

A slow flow sample of glutamate was taken overnight and in the morning before the rate was increased for experimental sampling. Neither the 3-hour or 24-hour slow flow glutamate samples were significantly different from controls for *xCT/sut* males in either the striatum or the cerebellum (One-tailed Mann-Whitney of glutamate levels striatum 24 hour, *xCT/sut* 14.4 ± 4.4µM n=5, *B6/SnJ* 17.7 ± 7.1µM n=4 p=0.37 Figure 5C; 3 hour striatum, *xCT/sut* 5.5 ± 4.3µM n=4, *B6/SnJ* 7.8 ± 3.6µM n=3 p=0.20 Figure 5D; cerebellum 24 hour, *xCT/sut* 10.9 ± 8.0µM n=3, *B6/SnJ* 6.4 ± 1.8µM n=5 p=0.50 Figure 5A; cerebellum 3 hour *xCT/sut* 3.4 ± 2.1µM n=2, *B6/SnJ* 13.2 ± 5.6µM n=3 p=0.27 Figure 5B).

Female slow flow glutamate samples were also not different from controls in either the striatum or cerebellum for the 3-hour or 24-hour time periods (One-tailed Mann-Whitney of glutamate levels striatum 24 hour, *xCT/sut* 12.3 ± 5.3µM n=5, *B6/SnJ* 16.4 ± 5.8µM n=6 p=0.40 Figure 6C; 3 hour striatum, *xCT/sut* 7.0 ± 4.5µM n=4, *B6/SnJ* 10.3 ± 3.3 µM n=5 p=0.21 Figure 6D; cerebellum 24 hour, *xCT/sut* 9.9 ± 4.8µM n=4, *B6/SnJ* 8.6 ± 2.2µM n=6 p=0.50 Figure 6A; cerebellum 3 hour *xCT/sut* 9.4 ± 4.0µM n=4, *B6/SnJ* 7.5 ± 1.6 n=5 p=0.37 Figure 6B). These

data indicate that neither *xCT/sut* males nor females had reduced glutamate in the slow flow samples in the striatum or cerebellum.

Male and Female Cerebellum

A

Striatum

B
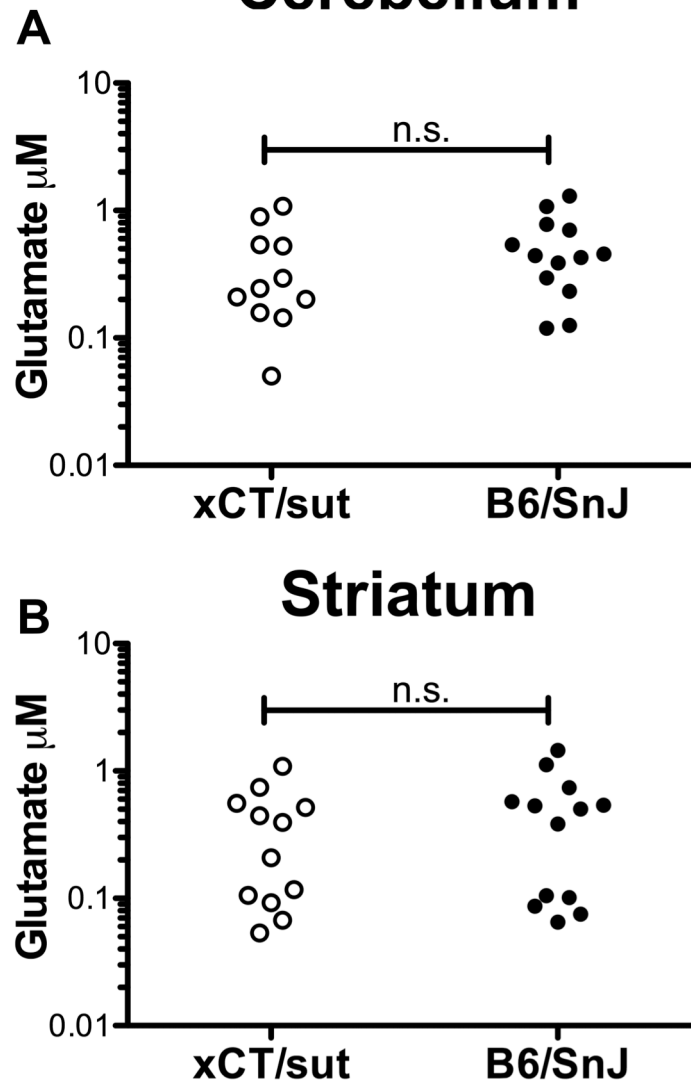
Figure 4 xCT/sut Mutant Glutamate Levels in the Cerebellum and Striatum

A, Glutamate levels in the striatum for males and females of xCT/sut and B6/SnJ genotypes (n=12 for xCT/sut, n=13 for B6/SnJ), B, Glutamate levels in the cerebellum for males and females of xCT/sut and B6/SnJ genotypes (n=11 for xCT/sut, n=13 for B6/SnJ).
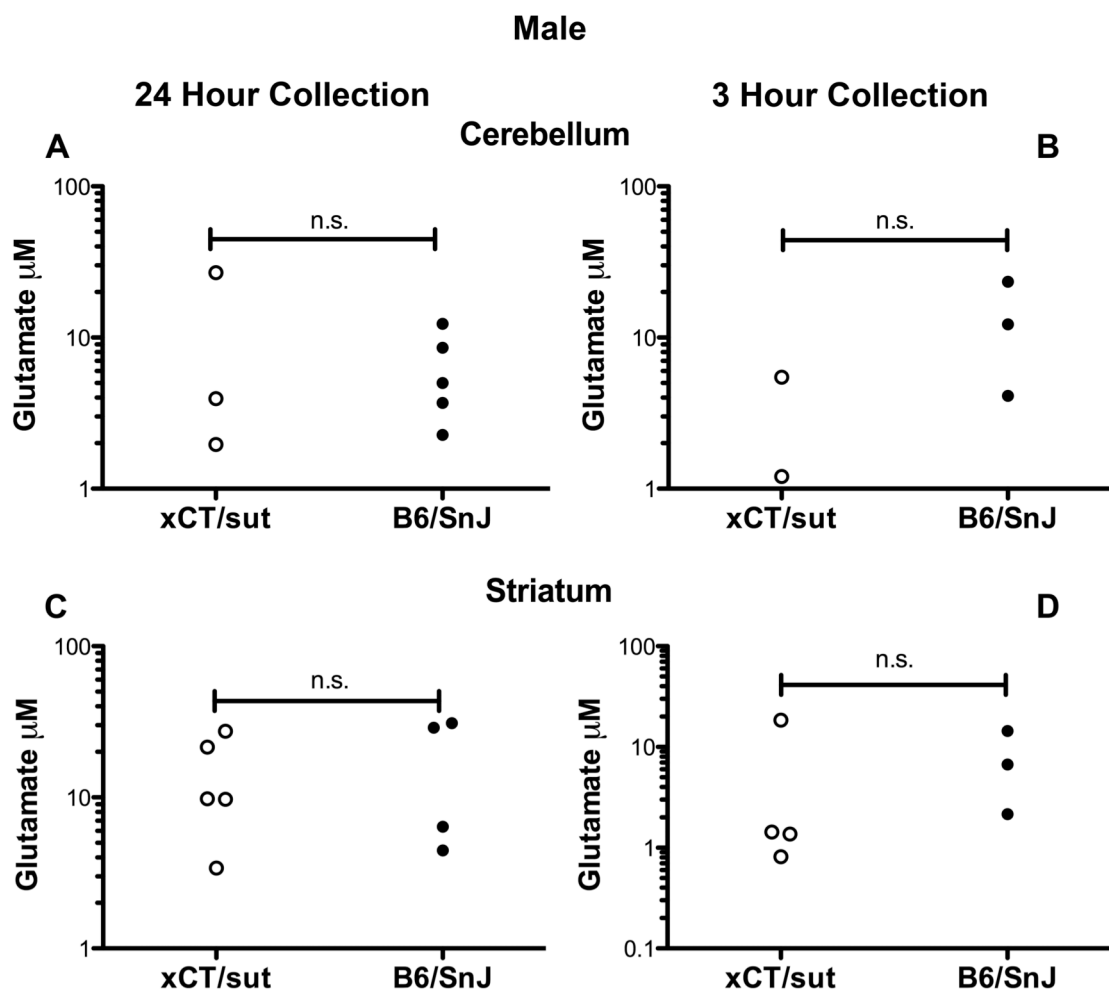
Figure 5 Male xCT/sut Mutant Slow Flow Glutamate Levels in the Cerebellum and Striatum

A, C, 24 hour collection measurements using a slow flow (0.07mL/min) from xCT/sut and B6/SnJ males in the cerebellum (A) and striatum (C). B,D, 3 hour slow flow (0.07mL/min) glutamate levels from xCT/sut and B6/SnJ males in the cerebellum (B) and striatum (D).

Figure 6 Female xCT/sut Mutant Slow Flow Glutamate Levels in the Cerebellum and Striatum

A, C, 24 hour collection measurements using a slow flow (0.07mL/min) from xCT/sut and B6/SnJ females in the cerebellum (A) and striatum (C). B,D, 3 hour slow flow (0.07mL/min) glutamate levels from xCT/sut and B6/SnJ females in the cerebellum (B) and striatum (D).
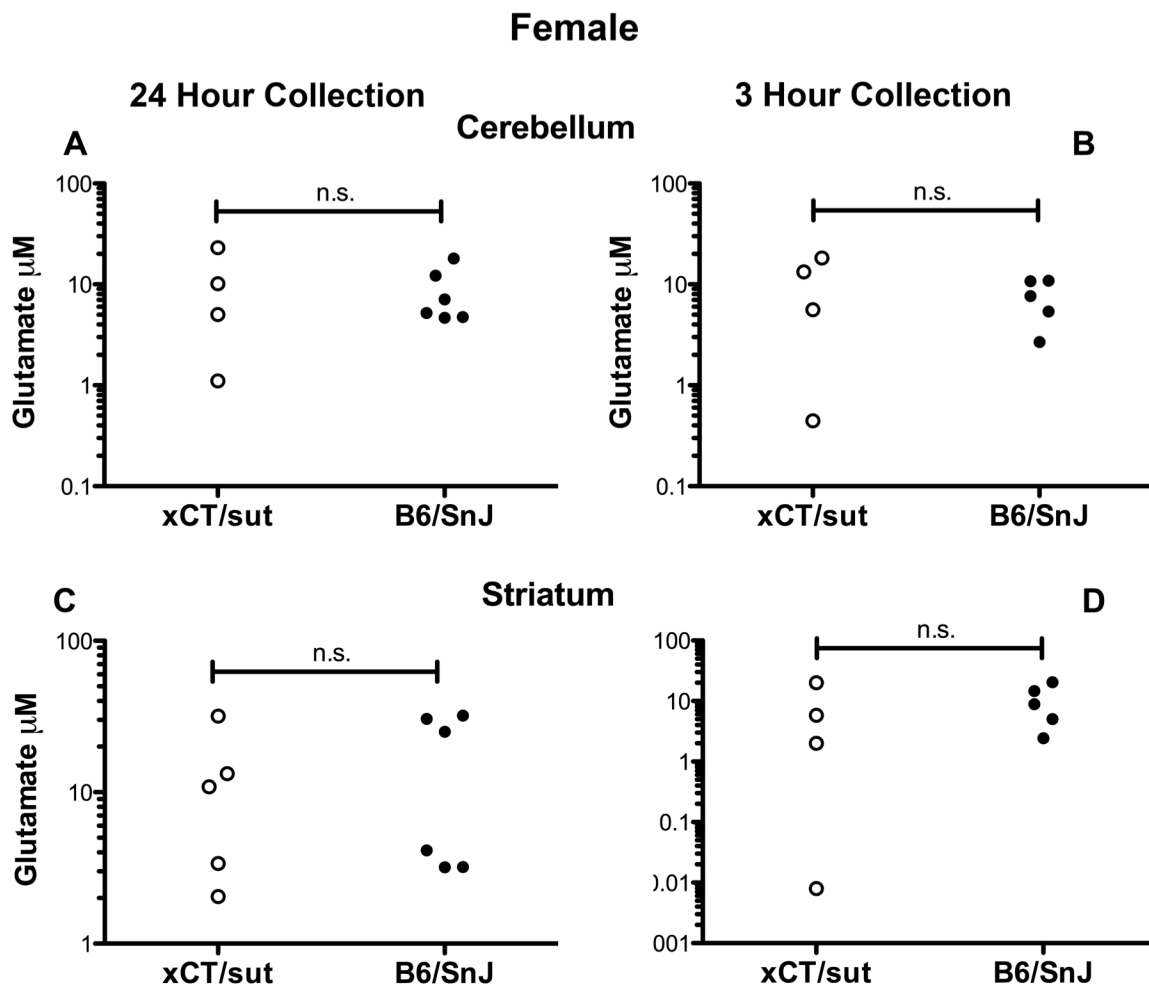
6.2.4 Aspartate levels in *sut* and *xCT/sut* animals in the striatum and cerebellum

Aspartate levels were used as a control for EAAT activity. *sut* males had similar aspartate levels compared to controls in both the striatum and cerebellum (One-tailed Mann-Whitney aspartate levels cerebellum *sut* 0.16 ± 0.11µM n=5, *SnJ* 0.07µM ± 0.03 n=5 p=0.27 Figure 7A; striatum, *sut* 0.4 ± 0.007µM n=4, *SnJ* 0.04 ± 0.01µM n=6 p=0.38 Figure 7B). Male *xCT/sut* animals had similar aspartate levels to their controls *B6/SnJ* in the striatum and the cerebellum (One-tailed Mann-Whitney aspartate levels cerebellum *xCT/sut* 0.77 ± 0.4µM n=5, *B6/SnJ* 0.30µM ± 0.06 n=6 p=0.47 Figure 8A; striatum, *xCT/sut* 0.2 ± 0.05µM n=6, *B6/SnJ* 0.81 ± 0.56µM n=6 p=0.35 Figure 8B). Similar to males, female *xCT/sut* mice also had similar aspartate levels in the striatum and cerebellum compared to their controls (One-tailed Mann-Whitney aspartate levels cerebellum *xCT/sut* 0.44 ± 0.21µM n=6, *B6/SnJ* 0.36 ± 0.14µM n=7 p=0.41 Figure 9A; striatum, *xCT/sut* 1.4 ± 0.97µM n=6, *B6/SnJ* 0.60 ± 0.28µM n=6 p=0.42 Figure 9B). Thus, neither *xCT/sut* males nor females had changes in aspartate levels in the striatum and cerebellum compared to their controls.
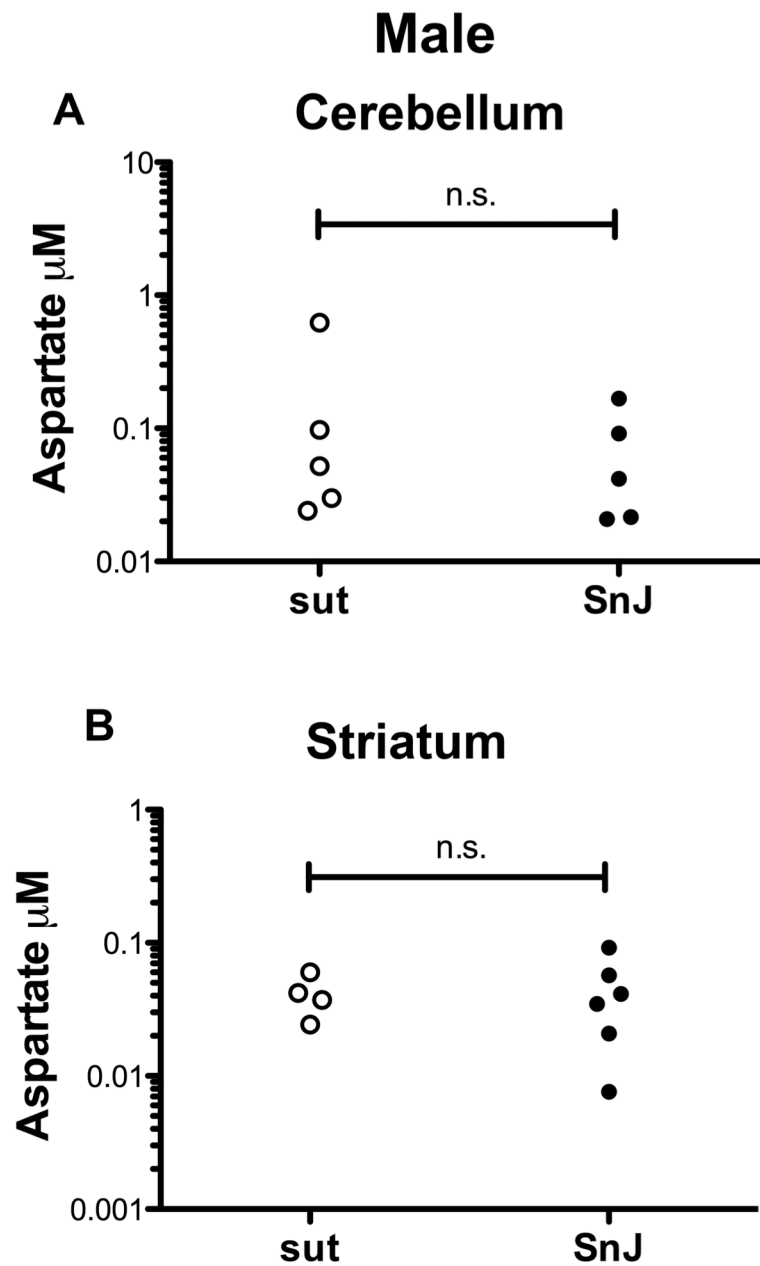
Figure 7 Male sut Mutant Aspartate Levels in the Cerebellum and Striatum.

A, aspartate levels of male sut and SnJ mice in the cerebellum. B, aspartate levels of male sut and SnJ mice in the striatum.

Figure 8 Male xCT/sut Mutant Aspartate Levels in the Cerebellum and Striatum.

A, aspartate levels of male xCT/sut and B6/SnJ mice in the cerebellum. B, aspartate levels of male xCT/sut and B6/SnJ mice in the striatum.

Female

A

Cerebellum

B
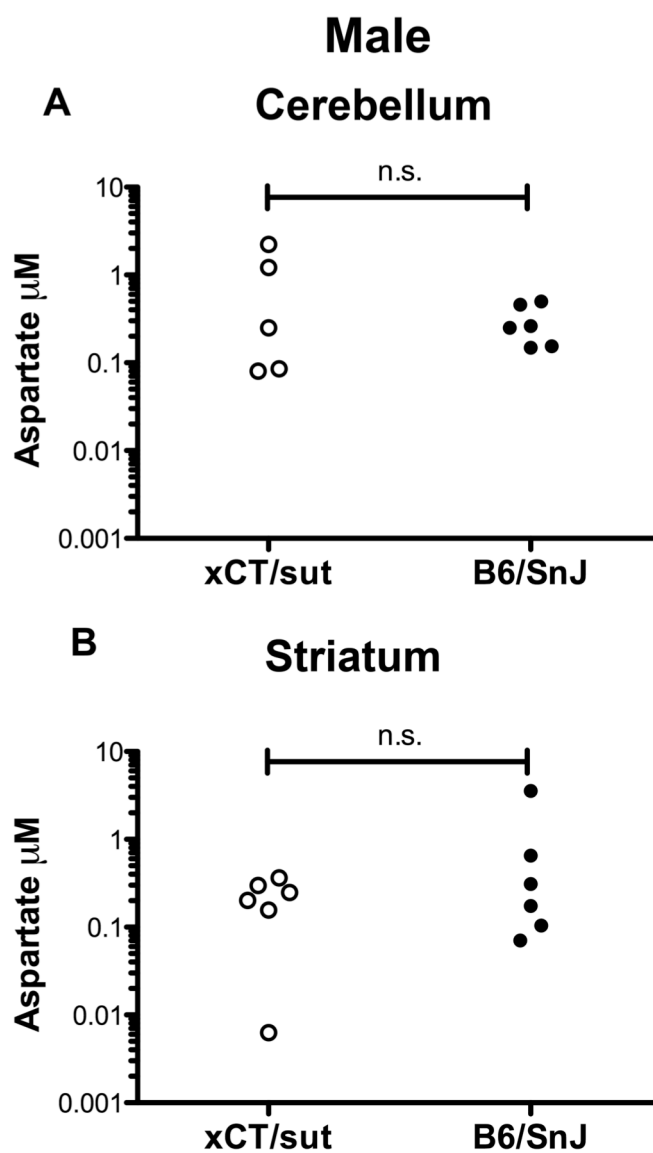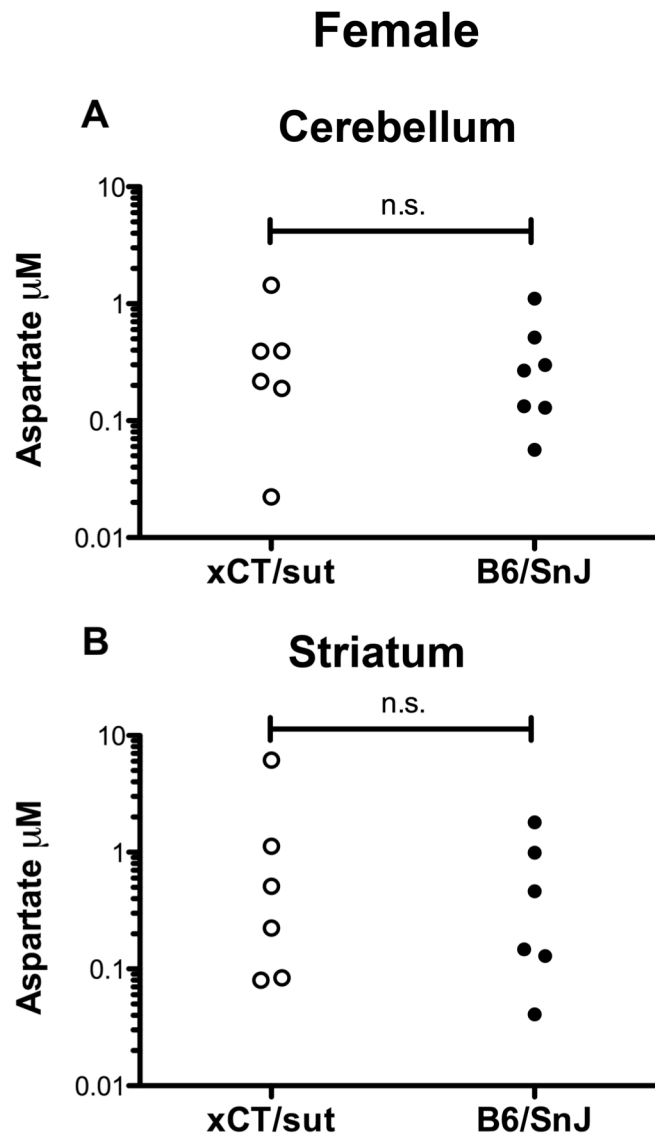
Striatum

Figure 9 Female xCT/sut Mutant Aspartate Levels in the Cerebellum and Striatum.

A, aspartate levels of female xCT/sut and B6/SnJ mice in the cerebellum. B, aspartate levels of female xCT/sut and B6/SnJ mice in the striatum.

6.3 Discussion

Glutamate levels were significantly lower in the striatum of *sut* mice compared to their controls, however cerebellum levels were not significantly different. Glutamate levels in the striatum and cerebellum of *xCT/sut* mice were not significantly lower than controls. There was no difference between glutamate levels of males and females of the *xCT/sut* cross. Even using the slow flow rate samples, which are thought to be a more accurate measure of total concentration of glutamate, there was no difference in glutamate levels in the *xCT/sut* cross (Chefer et al., 2001).

Consistent with data from *xCT* mice (Massie et al., 2011) there was a reduction in glutamate concentration in the striatum of *sut* mice. However, there was no reduction of glutamate levels in the striatum of the *xCT/sut* cross. There are several possible explanations for this; one is that the backgrounds of the two mutants may have some contribution to glutamate levels in the crosses. Also the number of animals measured in this study for the *sut* genotype are low, with more animals this phenotype could go away.

A second possible explanation for the lack of change in glutamate levels in these system xc- knockouts is that *sut* mice are not a full null knockout of system xc-. In fact, 3'-RACE data from Chintala et al suggests that *sut* mice may produce a truncated protein, despite seeing absence of *slc7a11* mRNA in Northern blots (Chintala et al., 2005). If *sut* mice are a functional hypomorph for system xc- this could explain both the behavioral data and the glutamate levels

seen in these brain areas (and the cross), particularly in the cerebellum where there was no a significant decrease in glutamate levels.

The third possible explanation is that due to the two different backgrounds, *xCT/sut* mice have some other compensatory mechanism that helps keep their glutamate levels at a more normal steady state. There are several ways in which extracellular glutamate may be regulated as discussed in more detail in the introduction. Some of which are enhanced synaptic release of glutamate, excitatory amino acid transporters, astrocytic release of glutamate, purinergic receptors, swelling-induced anion channels, and connexons (Malarkey and Parpura, 2008).

7 PROTEIN LEVEL CHANGES RELATED TO LOSS OF SYSTEM XC-

7.1 Introduction

One possible explanation for the *xCT/sut* mice not having a reduction in glutamate levels in the striatum is that *sut* mice are not a complete knockout of xCT protein. In fact according to 3'-RACE they may produce a shortened protein product since the mutation occurs in the last exon of the gene (Chintala et al., 2005). However the same study showed that there was no mRNA produced for the *Slc7a11* gene in *sut* knockouts (Chintala et al., 2005). A Western blot of xCT protein would confirm whether *sut* animals are complete protein-level system xc-knockouts.

Another way in which *sut* and *xCT/sut* animals might compensate for a lack of reduction in extracellular glutamate is through the excitatory amino acid transporters (EAATs). Excitatory amino acid transporters are located on neurons (EAAT-3) and glia (EAAT 1-2) and take up glutamate after synaptic release (Featherstone, 2010; Aoyama and Nakaki, 2013; Rodriguez et al., 2013). It has been shown that *xCT* mice do not have a change in the number of EAATs as measured by Western blots (De Bundel et al., 2011). However, *sut* mice are from a different background that may have some compensation for glutamate levels. If *sut* animals have a reduction in the number of EAATs as measured by Western blotting (and therefore less glutamate being taken up from the extracellular space), it could explain their normal levels of glutamate compared to controls in the cerebellum.

7.2 Results

7.2.1 xCT protein levels in system xc- knockout mice

It has never been shown that *sut* mice are a null mutant at the protein level for system xc-. Since *sut* mice appear to have a different behavioral phenotype than *xCT* mice, xCT protein levels in all three *slc7a11* mutant genotypes (*xCT*, *sut*, and *xCT/sut*) and their controls (*B6, SnJ, and B6/SnJ*) were measured to rule out the possibility that some of the differences might be attributable to incomplete elimination of xCT protein in *sut* mice.   The epitope for the antibody was for the initial 50 amino acid sequences to detect any conserved protein product that might occur in the *sut* mice.

As expected if *sut* mutants are protein nulls, Western blots showed no detectable protein in any of the three mutant genotypes, and there was no difference in xCT protein amount between the control backgrounds (One-way ANOVA with Tukey posttest of protein levels *B6/SnJ* 0.04 ± 0.01 n=3, *B6* 0.03 ± 0.02 n=3, *SnJ* 0.06 ± 0.04 n=3 Figure 1). All three xCT mutant strains used in this study (homozygous *sut*, homozygous *xCT*, and *xCT/sut*) do not produce any xCT protein measured by Western blotting.

Figure 1 xCT protein levels in knockouts and backgrounds.

An example Western blot and quantification of protein levels in males and females combined.
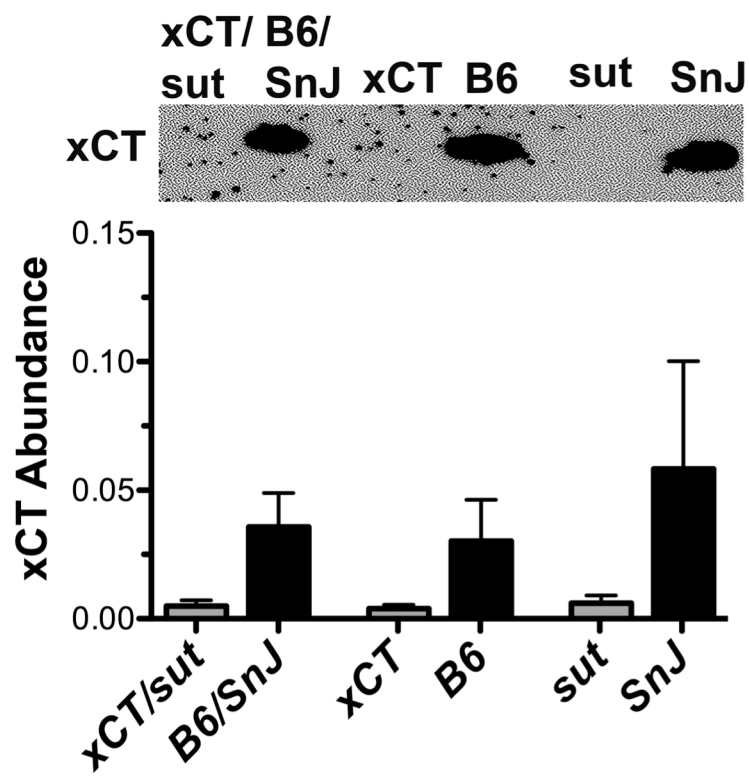
7.2.2 EAAT-1 levels in system xc- knockouts

To test whether levels of the EAATs were different in the three knockout strains, Western blots were performed and normalized to an actin control. EAAT-1 levels were not different between the system xc- knockouts and their controls when males and females were combined (Two-tailed t-test of EAAT-1 abundance *xCT/sut* 0.16 ± 0.02 n=6, *B6/SnJ* 0.16 ± 0.03 n=6 p=0.95, *xCT* 0.17 ± 0.04 n=6, *B6* 0.20 ± 0.03 n=6 p=0.63, *sut* 0.18 ± 0.03 n=6, *SnJ* 0.13 ± 0.03 n=6 p=0.25 Figure 2B). Males and females were not significantly different from each other (Two-way ANOVA with Bonferroni posttest comparison across each genotype between sexes in for EAAT-1 p>0.05).

When data was separated by sex there was still no difference in EAAT-1 levels between any of the system xc- knockouts and their controls (Two-tailed t-test of EAAT-1 abundance, males *xCT/sut* 0.14 ± 0.04 n=3, *B6/SnJ* 0.12 ± 0.03 n=3 p=0.64, *xCT* 0.13 ± 0.03 n=3, *B6* 0.15 ± 0.04 n=3 p=0.71, *sut* 0.17 ± 0.05 n=3, *SnJ* 0.12 ± 0.05 n=3 p=0.51 Figure 2C; females *xCT/sut* 0.17 ± 0.02 n=3, *B6/SnJ* 0.20 ± 0.05 n=3 p=0.63, *xCT* 0.21 ± 0.07 n=3, *B6* 0.24 ± 0.04 n=3 p=0.72, *sut* 0.19 ± 0.03 n=3, *SnJ* 0.14 ± 0.04 n=3, Figure 2D). Neither males nor females of any of the system xc- mutants have changes in EAAT-1 protein abundance.

Figure 2 Changes in EAAT-1 expression in xCT knockout mice

A, A representative Western blot showing EAAT-1 (~65kDa) and actin (~45kDa).
B, Combined quantitative data for males and females for
EAAT-2 abundance (n=6/genotype) C, D, Quantitative data for female and male
Western blots in EAAT-1 abundance (n=3/genotype). Abundance quanitfied as
relative to actin.
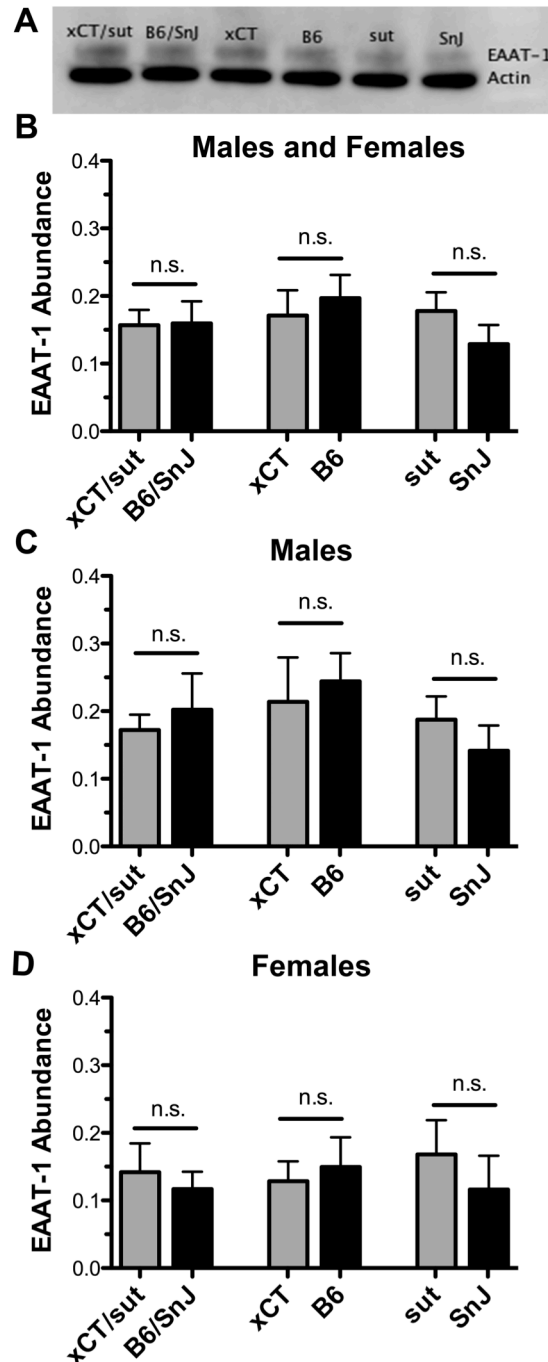
7.2.3 EAAT-2 levels in system xc- knockouts

EAAT-2 abundance was also not different between system xc- knockouts and their controls when data for males and females was combined (Two-tailed t-test EAAT-2 abundance *xCT/sut* 1.90 ± 0.14 n=6, *B6/SnJ* 2.17 ± 0.42 n=6 p=0.56, *xCT* 1.9 ± 0.25 n=6, *B6* 2.03 ± 0.28 n=6 p=0.70, *sut* 1.60 ± 0.25 n=6, *SnJ* 1.65 ± 0.31 n=6 p=0.90, Figure 3B). Males and females were not significantly different from each other (Two-way ANOVA with Bonferroni posttest comparison across each genotype between sexes for EAAT-2 p>0.05).

When the sexes were analyzed separately there was also no difference in EAAT-2 expression compared to their controls (Two-tailed t-test EAAT-2 abundance males, *xCT/sut* 1.9 ± 0.17 n=3, *B6/SnJ* 2.7 ± 0.73 n=3 p=0.36, *xCT* 2.0 ± 0.50 n=3, *B6* 2.2 ± 0.51 n=3 p=0.75, *sut* 1.4 ± 0.38 n=3, *SnJ* 1.4 ± 0.19 n=3 p=0.93, Figure 3C). Females, *xCT/sut* 1.9 ± 0.26 n=3, *B6/SnJ* 1.7 ± 0.32 n=3 p=0.59, *xCT* 1.8 ± 0.22 n=3, B6 1.8 ± 0.31 n=3 p=0.89, *sut* 1.8 ± 0.36 n=3, *SnJ* 1.9 ± 0.60 n=3 p=0.85, Figure 3D). In summary, neither system xc- mutant males nor females showed a change in the abundance of EAAT-2.

Figure 3 Changes in EAAT-2 expression in xCT knockout mice

A, A representative Western blot showing EAAT-2 (~65kDa) and actin
(~45kDa). B, Combined quantitative data for males and females for
EAAT-2 abundance (n=6/genotype) C, D, Quantitative data for female
and male Western blots in EAAT-2 abundance (n=3/genotype).
Abundance quanitfied as relative to actin.
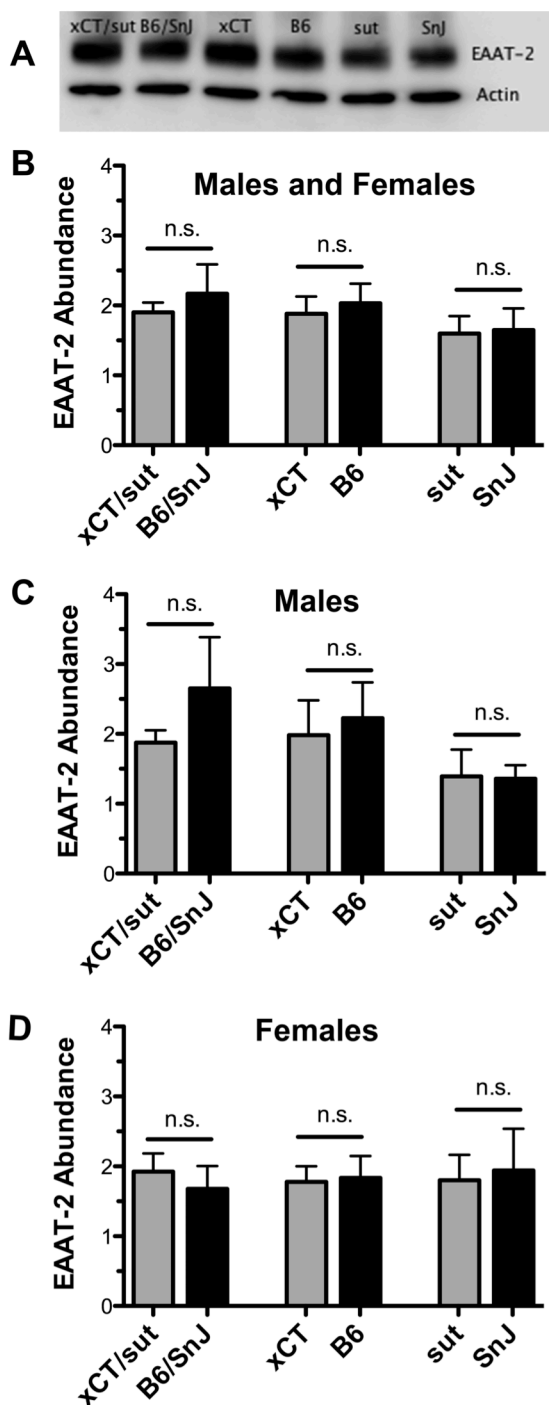
7.2.4 EAAT-3 levels in system xc- knockouts

EAAT-3, similarly to EAAT-1 and EAAT-2, is not different between the three system xc- mutants when males and females are combined (Two-tailed t-test EAAT-3 abundance *xCT/sut* 0.035 ± 0.01 n=6 *B6/SnJ* 0.027 ± 0.012 n=6 p=0.66, *xCT* 0.020 ± 0.0067 n=6, *B6* 0.031 ± 0.013 n=6 p=0.47, *sut* 0.048 ± 0.019 n=6, *SnJ* 0.037 ± 0.019 n=6 p=0.69, Figure 4B). Males and females were also not different from each other for each of the system xc- mutants (Two-way ANOVA with Bonferroni posttest comparison across each genotype between sexes for EAAT-3 p>0.05).

When the sexes are separated out, there is also no difference in EAAT-3 abundance between the system xc- mutants and their backgrounds (Two-tailed t-test EAAT-3 abundance males, *xCT/sut* 0.027 ± 0.0035 n=3, *B6/SnJ* 0.027 ± 0.015 n=3 p=0.99, *xCT* 0.029 ± 0.011 n=3, *B6* 0.036 ± 0.020 n=3 p=0.76, *sut* 0.046 ± 0.014 n=3, *SnJ* 0.029 ± 0.0023 n=3, Figure 4C; females, *xCT/sut* 0.042 ± 0.022 n=3, *B6/SnJ* 0.028 ± 0.023 n=3 p=0.67, *xCT* 0.011 ± 0.0039 n=3, *B6* 0.025 ± 0.020 n=3 p=0.52, *sut* 0.050 ± 0.041 n=3, *SnJ* 0.045 ± 0.042 n=3 p=0.93, Figure 4D). Thus, neither male nor female system xc- mutants had differences in abundance of EAAT-3.
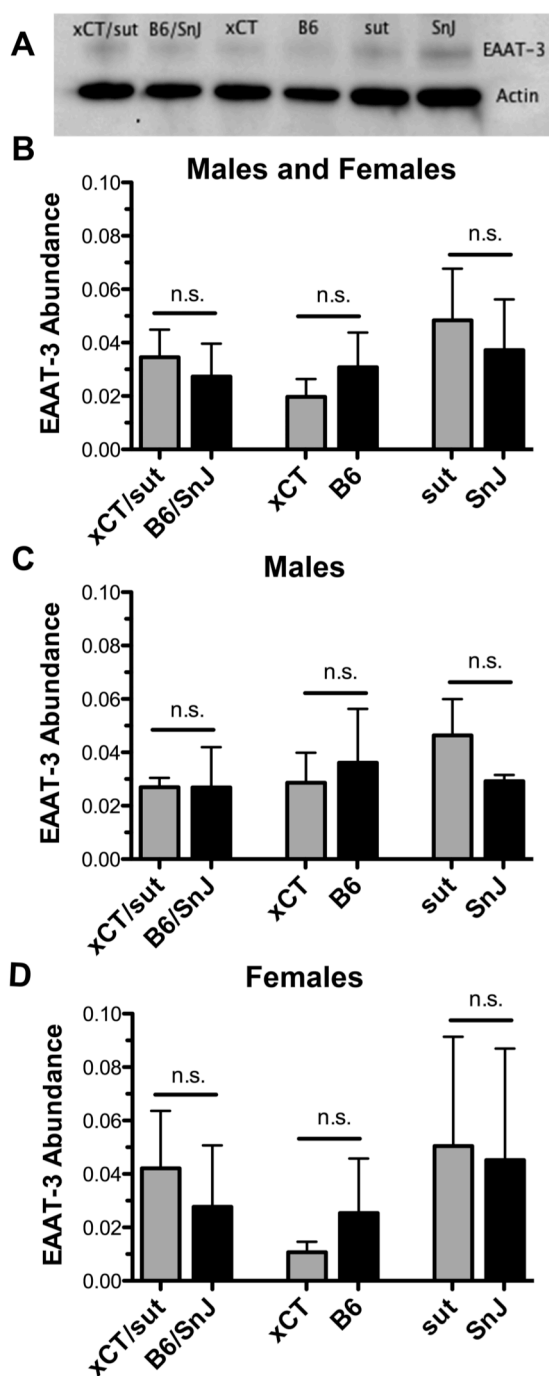
Figure 4 Changes in EAAT-3 expression in xCT knockout mice

A, A representative Western blot showing EAAT-3 (~65kDa) and actin (~45kDa). B, Combined quantitative data for males and females for EAAT-2 abundance (n=6/genotype) C, D, Quantitative data for female and male Western blots in EAAT-3 abundance (n=3/genotype). Abundance quanitfied as relative to actin.

7.3 Discussion

It does appear that *sut* mutants are indeed protein level knockouts of system xc-. Also, consistent with data from Ann Massie's lab, there are no changes in EAAT1-3 abundance in the brains of any of the system xc- mutants (De Bundel et al., 2011). Furthermore, changes in abundance of EAAT proteins cannot account for the normal levels of extracellular glutamate seen in *sut* and *xCT/sut* cross mice in the cerebellum or the striatum of *xCT/sut* animals. One limitation to this study is that whole brain homogenates were used as the protein source for the Western blots.

Perhaps there are more regional specific changes in protein levels in these transporters. Indeed it is known that each EAAT while common to the entire brain, are up regulated in certain areas and help control glutamate uptake in those areas (Watase et al., 1998; Verrey et al., 2004; Dahlin et al., 2009; Holmseth et al., 2009; SARAC et al., 2009; Massie et al., 2010; Kanai et al., 2013). A more selective approach of measuring protein levels in just the striatum and cerebellum for each EAAT might elucidate whether they are impacting glutamate levels locally in those areas in the system xc- knockouts. Function of EAATs rather than protein levels could also be the compensatory mechanism by which glutamate levels are normalized.

8 RELATIONSHIP BETWEEN ESTRUS AND XCT KNOCKOUT BEHAVIOR

8.1 <u>Introduction</u>

System xc- mutants do not have a consistent behavioral phenotype suggesting that any behavioral deficits that are related to loss of system xc- expression are probably very subtle in nature. *sut* system xc- mutants did have some behavioral changes compared to their controls *SnJ*. *sut* males had a reduction in arm choices in the four-arm and three-arm spontaneous maze and one of the arms of the delayed task. Males also had a reduction in spontaneous alternation ability in the three-arm maze. Unlike females however they made fewer grid crossings in the open field maze suggesting that the reduction in arm choices in the spontaneous alternation maze could be due to a movement deficit. Female *sut* mice also had a reduction in arm choices in the four-arm task and the delayed task, but not in the three-arm maze. As mentioned before, females did not have a behavioral phenotype in the open-field maze. Perhaps behavior in *sut* females can be explained by something other than a motor deficit such as working memory changes related to the estrus cycle.

There have been a few studies that show a relationship between hormonal changes during the estrus cycle and the spontaneous alternation task (Miller et al., 1999; Korol et al., 2004; Frye and Walf, 2008; Walf et al., 2009). Perhaps estrogen binds to system xc- and can regulate and change behavior in controls over the different stages of estrus compared to the system xc- mutants. There could also be differences between the backgrounds of the *sut* and *xCT* mouse

strains during the estrus cycle and how they perform in the spontaneous alternation task. Both whether *sut* behavior can be explained by changes in the estrus cycle, and whether the backgrounds differ throughout the cycle will be analyzed in this section.

8.2 Results

8.2.1 Estrus stage and spontaneous alternation ability in *sut* and *xCT*

Samples from each mouse were taken prior to testing in the four-arm spontaneous alternation task and then later it was determined what stage of the estrus cycle animals were in. Figure 1 shows an example of what various cell types are present in each stage of estrus. Neither *sut* nor *xCT* mice differed from their controls during the estrus cycle in their percent alternations in the four-arm task (Two-way ANOVA without matching % alternations proestrus, *sut* 17.1 ± 4.7% n=15, *SnJ* 19.3 ± 5.0% n=10 p>0.05, estrus *sut* 13.3 ± 3.9% n=22, *SnJ* 14.5 ± 3.3% n=16 p>0.05, metestrus *sut* 11.8 ± 4.7% n=12, *SnJ* 18.7 ± 4.3% n=14 p>0.05, diestrus *sut* 10.0 ± 4.0% n=12, *SnJ* 10.5 ± 4.3% n=9 p>0.05 Figure 2A; proestrus, *xCT* 28.3 ± 3.0% n=11, *B6* 24.2 ± 3.3% n=10 p>0.05, estrus *xCT* 28.7 ± 2.8% n=11, *B6* 26.7 ± 3.2% n=10 p>0.05, metestrus *xCT* 24.8 ± 1.8% n=10, *B6* 29.6 ± 2.9% n=10 p>0.05, diestrus *xCT* 31.0 ± 2.6%, *B6* 30.1 ± 4.3% n=10 p>0.05, Figure 2B). Neither system xc- mutant *sut* nor *xCT* showed a deficit compared to their controls with spontaneous alternation percent during the stages of estrus.
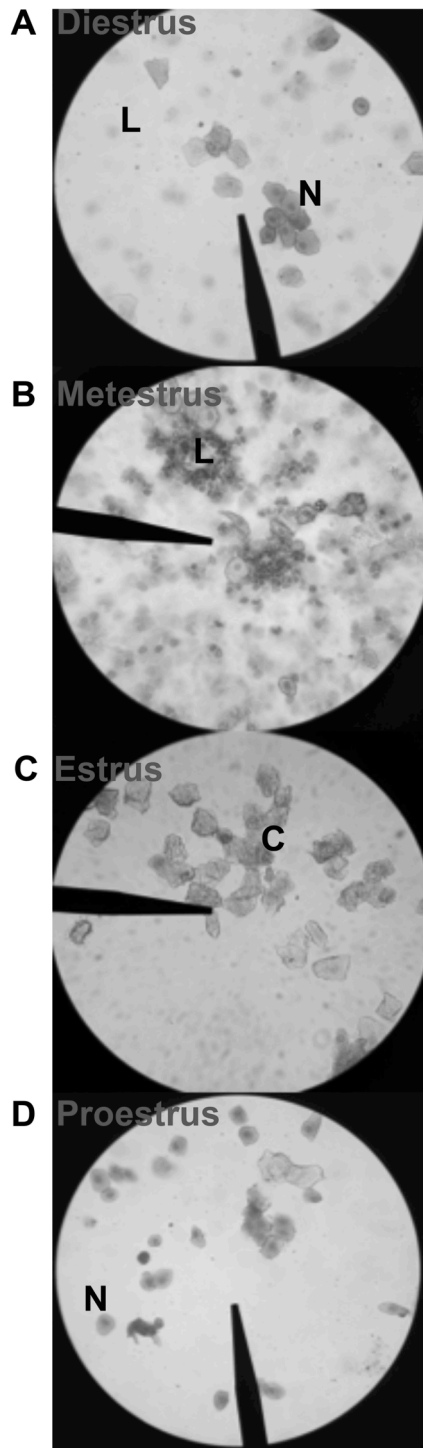
Figure 1 Examples of estrus stage results from female mice

A, diestrus, a stage that an even mix between leukocytes (L) and nucleated cells (N), B, metestrus, stage that is predominantly leukocytes (L) with some nucleated cells and cornified cells (C). C, estrus, stage is mostly cornified cells (C). D, proestrus, mostly nucleated cells (N).
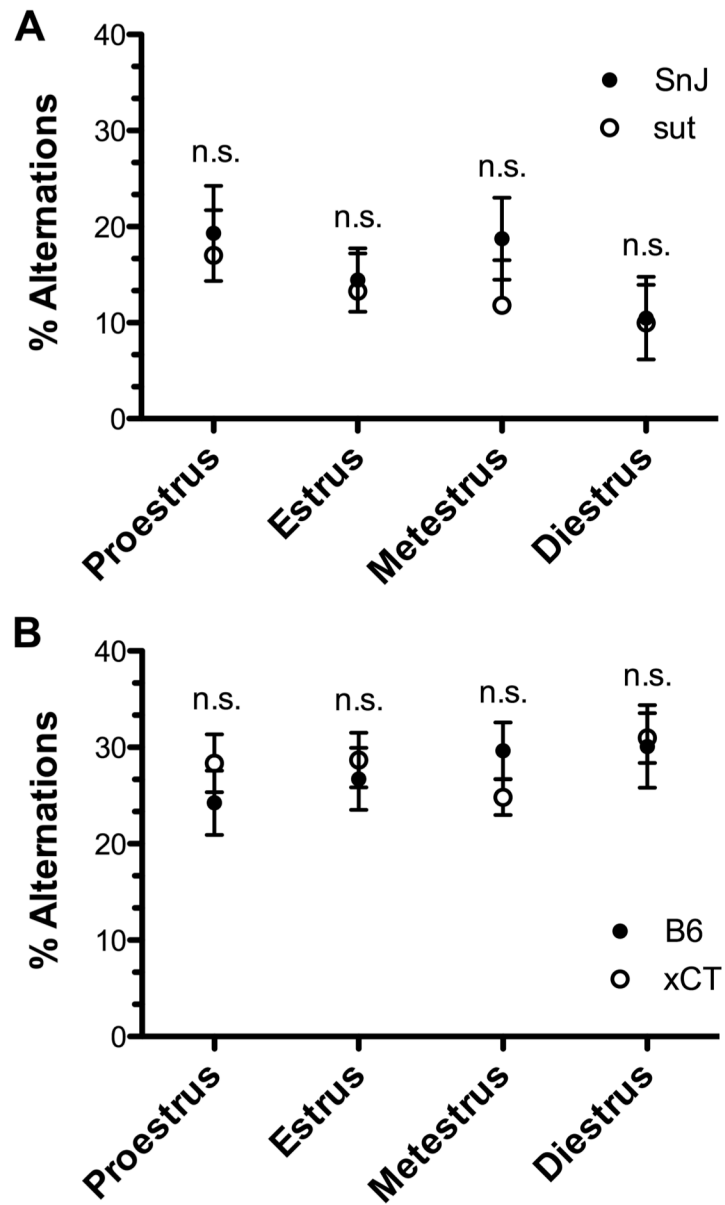
Figure 2 xCT Mutants Performance in a Spontaneous Alternation Task Across the Four Stages of Estrus.

A, comparison of alternation ability in sut and their controls (SnJ). B, comparison of alternation ability in xCT and their controls (B6) (n= at least 10 for each genotype/stage).

Neither *sut* nor *xCT* females were different than their controls in the number of arm choices they made in the four-arm spontaneous alternation task across the stages of the estrus cycle (Two-way ANOVA without matching number of arm choices proestrus, *sut* 14.7 ± 3.4 choices n=15, *SnJ* 21.0 ± 4.5 choices n=10 p>0.05, estrus *sut* 11.8 ± 2.4 choices n=22, *SnJ* 19.3 ± 3.1 choices n=16 p>0.05, metestrus *sut* 10.6 ± 3.9 choices n=12, *SnJ* 16.7 ± 2.9 choices n=14 p>0.05, diestrus *sut* 10.6 ± 3.9 choices n=12, *SnJ* 10.9 ± 4.2 choices n=9 p>0.05 Figure 3A; proestrus, *xCT* 28.4 ± 1.2 choices n=11, *B6* 31.4 ± 1.7 choices n=10 p>0.05, estrus *xCT* 26.5 ± 1.3 choices n=11, *B6* 30.1 ± 2.3 choices n=10 p>0.05, metestrus *xCT* 31.9 ± 2.6 choices n=10, *B6* 30.6 ± 2.2 choices n=10 p>0.05, diestrus *xCT* 31.2 ± 2.2 choices, *B6* 30.9 ± 2.3 choices n=10 p>0.05, Figure 3B). Females of both genotypes do not differ from their controls in the number of arm choices they make in this task during the different stages of estrus.

8.2.2 Comparison of background strains and two mutants

Analyzing the same data but comparing the two mutants to each other and the backgrounds to each other, it is clear that there is a difference between the backgrounds (and subsequently the mutants) during the stages of estrus in their spontaneous alternation percent (Two-way ANOVA without matching % alternations proestrus, *B6* 24.2 ± 3.3% n=10, *SnJ* 19.3 ± 5.0% n=10 p>0.05, estrus, *B6* 26.7 ± 3.2% n=10, *SnJ* 14.5 ± 3.3% n=16 p>0.05, metestrus, *B6* 29.6 ± 2.9% n=10, *SnJ* 18.7 ± 4.3% n=14 p>0.05, diestrus, *B6* 30.1 ± 4.3% n=10, *SnJ*

10.5 ± 4.3% n=9 p<0.01 Figure 4A; proestrus, *sut* 17.1 ± 4.7% n=15, *xCT* 28.3 ±

3.0% n=11, p>0.05, estrus *sut* 13.3 ± 3.9% n=22, *xCT* 28.7 ± 2.8% n=11 p<0.05,

metestrus *sut* 11.8 ± 4.7% n=12, *xCT* 24.8 ± 1.8% n=10, p>0.05, diestrus *sut*

10.0 ± 4.0% n=12, *xCT* 31.0 ± 2.6%, p<0.01, Figure 4B). There is a reduction in

the percent alternations for *SnJ* females compared to *B6* females during diestrus.

There is a reduction in *sut* alternation percent compared to *xCT* during estrus

and diestrus. These results suggest that the backgrounds perform differently

during the different stages of estrus when compared to each other in the percent

alternation ability.
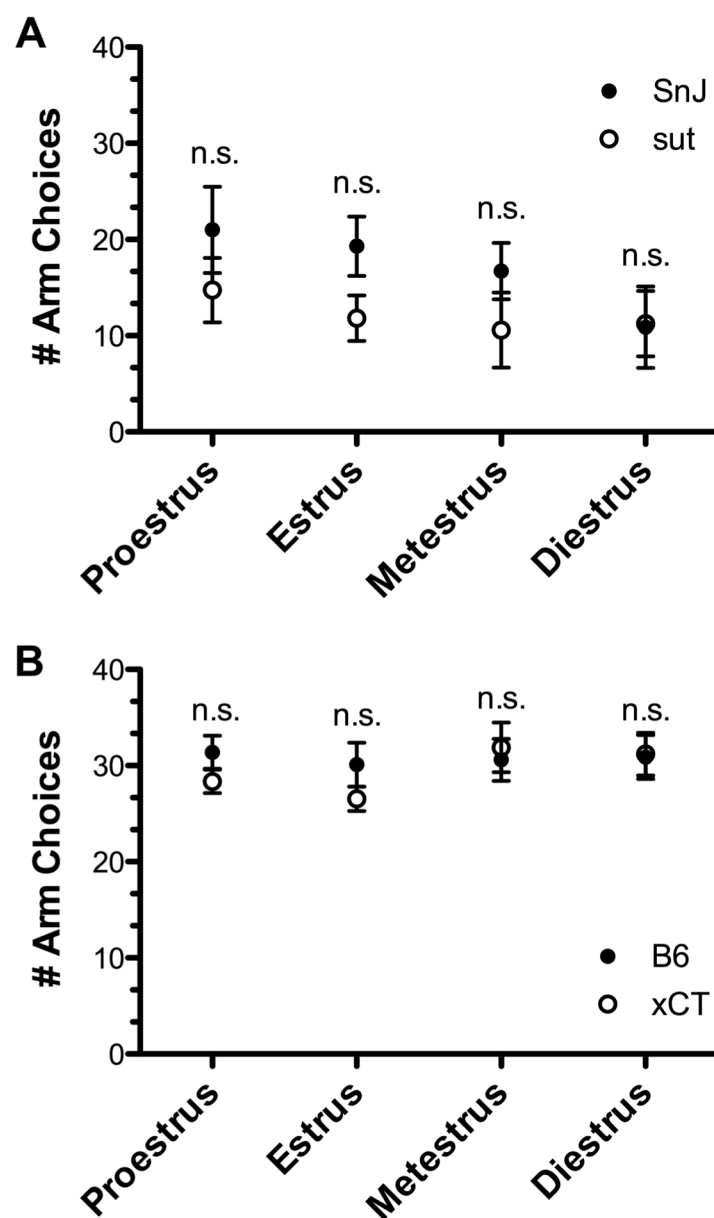
Figure 3 xCT Mutants Number of Arm Choices in a Spontaneous Alternation Task across the Four Stages of Estrus.

A, comparison of number of arm choices in sut and their controls (SnJ). B, comparison of number of arm choices in xCT and their controls (B6) (n= at least 10 for each genotype/stage).
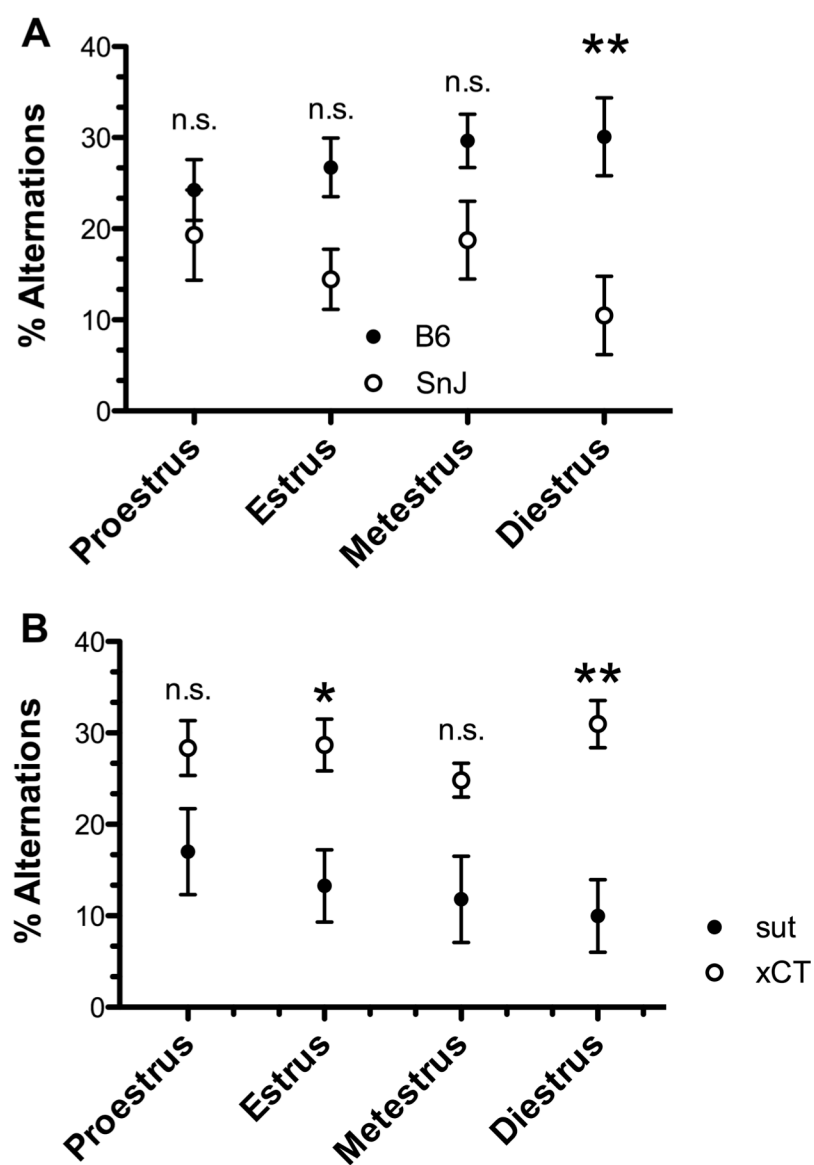
Figure 4 xCT Mutants Performance in a Spontaneous Alternation Task across the Four Stages of Estrus and Comparison of the two backgrounds and mutants.

A, comparison of alternation ability in SnJ and B6. B, comparison of alternation ability in xCT and sut (n= at least 10 for each genotype/stage *=p<0.05, **=p<0.01).

More significantly, *SnJ* animals have a reduction in the number of arm choices compared to the other background *B6* during three stages of the cycle; estrus, metestrus and diestrus (Two-way ANOVA without matching number of arm choices proestrus, *B6* 31.4 ± 1.7 choices n=10, *SnJ* 21.0 ± 4.5 choices n=10 p>0.05, estrus *B6* 30.1 ± 2.3 choices n=10, *SnJ* 19.3 ± 3.1 choices n=16 p<0.05, metestrus *B6* 30.6 ± 2.2 choices n=10, *SnJ* 16.7 ± 2.9 choices n=14 p<0.01, diestrus, *SnJ* 10.9 ± 4.2 choices n=9 p<0.001 Figure 5A). Similarly when the two mutants were compared, *sut* mice made fewer arm choices through all stages of the estrus cycle (Two-way ANOVA without matching number of arm choices proestrus, *sut* 14.7 ± 3.4 choices n=15, *xCT* 28.4 ± 1.2 choices n=11, p<0.01, estrus *sut* 11.8 ± 2.4 choices n=22, *xCT* 26.5 ± 1.3 choices n=11, p<0.001, metestrus *sut* 10.6 ± 3.9 choices n=12, *xCT* 31.9 ± 2.6 choices n=10, p<0.001, diestrus *sut* 10.6 ± 3.9 choices n=12, *xCT* 31.2 ± 2.2 choices, *B6* 30.9 ± 2.3 choices n=10 p<0.001, Figure 5B). These data are indicative that there are differences in the backgrounds performance in the number of arm choices during the four-arm spontaneous alternation task.

When comparing across the different stages in the same genotype, i.e. *SnJ* estrus compared to *SnJ* diestrus, there were no differences in percent spontaneous alternation or number of arm choices at any of the stages of estrus within any of the genotypes (One-way ANOVA with Tukey's post-test). Data was also collected for estrus stage in several of the experiments in Chapter 5, that data is show in the Appendix since the numbers were too low for statistical analysis.

Figure 5 xCT Mutants Number of Arm Choices in a Spontaneous Alternation Task across the Four Stages of Estrus and Comparison of the two backgrounds and mutants.

A, comparison of number of arm choices in SnJ and B6. B, comparison of number of arm choices in xCT and sut (n= at least 10 for each genotype/stage *=p<0.05, **=p<0.01, ***=p<0.001).
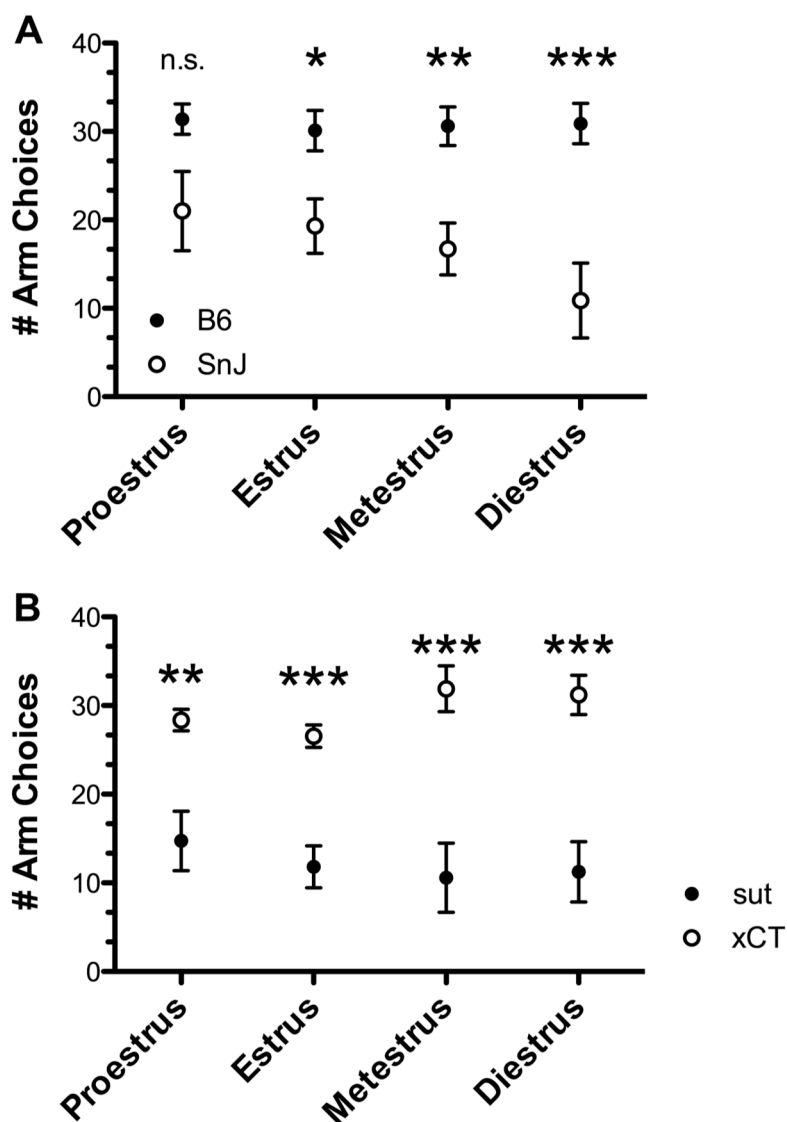
8.3 <u>Discussion</u>

While it appears that there are no differences during the estrus cycle that can be attributed to system xc- (by looking at changes between the mutant and its control Figures 2-3), there are differences between the backgrounds during the estrus cycle (Figures 4-5). Since backgrounds differ during the estrus cycle it is possible that female *sut* mutant behavior may be attributed to some other mutations in the *SnJ* background, which along with lacking system xc-, lead to changes in behavior. It has been shown that the background genotype of animals can have a significant impact on behavior and that multiple backgrounds should be used whenever possible for experiments similar to studies in other genetic models such as *C. elegans* and *Drosophila melanogaster* (McFadyen et al., 2003; McCutcheon et al., 2008).

9   DISCUSSION AND FUTURE STUDIES

9.1 Behavioral analysis of system xc- mutants

The aim of this study was to characterize behavior in system xc- mutants. It was predicted there would be some significant behavioral effects of system xc-loss since this transporter affects glutamate receptor number, oxidative stress, cell death and metabotropic receptor tone. The present study found that there were some significant behavioral deficits in *sut* system xc- mutants, however these behaviors could not be duplicated in the other system xc- mutant *xCT* or the cross *xCT/sut.*

Specifically, male *sut* males had a reduction in arm choices in the four arm task, a reduced number of grid crossings in the open field, a reduction in number of arm choices and percent alternations in the three-arm maze, and a reduction in number of arm choices in one of the arms in the delayed spontaneous alternation task compared to their controls *SnJ*. *sut* females had similar deficits to *sut* males, but did not have a reduced number of grid crossings in the open field or reduced arm choices in the three-arm task compared to *SnJ* mice. In contrast, *xCT* mice and *xCT/sut* mice did not have any deficits in these tasks compared to their controls *B6* or *B6/SnJ*.

Contrary to my findings, Dr. Massie's group found that young *xCT* mice had a reduction in spontaneous alternation ability similar to what we found in *sut* mice in the three-arm maze. However, older *xCT* mice did not have this deficit

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(De Bundel et al., 2011).   Perhaps there are some age-dependent changes in behavior in these mice.

Li et al found that *sut* mice had impaired fear conditioning and passive avoidance compared to their controls (Li et al., 2012b). This is consistent with the memory deficits that I saw in the spontaneous alternation task. The deficits I saw in the open-field maze could also explain the reduction in freezing seen in *sut* mice in the fear conditioning experiments if they have motor impairments.

Another possible reason for the discrepancy in behavioral deficits seen in *sut* mice compared to *xCT* is that they come from very different background strains. Each background strain has its own mutations inherent to the strain. When these background mutations are then combined with the system xc- mutation it may exacerbate or alleviate the effects of the system xc- mutation. For example, as seen in female mice, the two background strains behaved very differently in the four-arm spontaneous alternation task across the different stages of estrus (Chapter 8, Figure 4 and Figure 5). Studies have shown that the background of a mouse strain can have impacts on neurogenesis and locomotion across backgrounds in a neurokinin-1 knockout (McCutcheon et al., 2008). It has also been shown that there are differences in background strain's ability to perform on a rotarod task (McFadyen et al., 2003). There are few studies that compare mutations across multiple background strains, so it is unclear how important strain effects might be.

It is still possible that there are behavioral consequences to loss of system xc-. In the *genderblind* flies, unless you are specifically looking at courtship

behavior, the bisexual phenotype of *gb* male flies would have been easily missed, and all other assays of *gb* flies appeared normal (Grosjean et al., 2007). Thus, there could be other systems of behavior which were not assessed in these experiments that system xc- may have a role in regulating.

One possible area to pursue is the effect of system xc- loss on sleep in mice. It has been shown that system xc- is upregulated in the cerebral spinal fluid (CSF) and may control glutamate levels in this area (Pow, 2001; Ohtsuki, 2004; Burdo et al., 2006). A recent study has shown that the CSF exchanges fluids with the interstitial space much more during sleep perhaps as a mechanism to clear neurotoxic waste products accumulated when animals are awake (Xie et al., 2013). It has also been shown that glutamate levels decrease in the prefrontal and motor cortex in rats during non-REM sleep, and steadily increase in awake animals (Dash et al., 2009; Vyazovskiy et al., 2009). This is consistent with studies that show that synaptic strength is decreased during sleep states and that sleep may play a role in synaptic homeostasis (Tononi and Cirelli, 2003; 2006; Halassa et al., 2009; Liu et al., 2010).

CSF glutamate levels that are determined by system xc- activity could quickly and tightly regulate glutamatergic synaptic strength between sleep and wake periods. System xc- may silence ionotropic receptors during sleep by influxing glutamate from the CSF into the extracellular space. It would be expected then that system xc- mice would sleep less or have shorter bouts of sleep compared to their controls.

In summary, while there was no consistent behavioral effect of loss of system xc- in this study, there are still possible subtle behavioral changes in system xc- mice. Sleep behavior, which was not studied in these experiments, may be regulated by system xc- and would be interesting to study in the future.

9.2 Glutamate level analysis in system xc- mutants

Glutamate levels were not found to be significantly decreased in either male or female *xCT/sut* cross mice in the striatum or cerebellum and in *sut* males in the cerebellum. Whereas, *sut* males did have a reduction of glutamate levels in the striatum, which is consistent with what was seen in the *xCT* mice (Massie et al., 2011). However, the transheterozygote cross did not show a reduction in of glutamate levels in the striatum indicating that system xc- is probably not the only major regulator of glutamate in this area. There also have been no studies that show a reduction in extracellular glutamate in the cerebellum in other system xc- knockouts. Thus, system xc- may not be an important regulator of extracellular glutamate in the cerebellum. It is also possible that system xc- is not a global regulator of extracellular glutamate in all brain areas. For example, Dr. Massie has shown no reduction in extracellular glutamate levels in the prefrontal cortex of *xCT* mice (unpublished data).

One pitfall to using microdialysis studies for the measurement of brain metabolites is that it can cause stress to the brain with the introduction of the probe (Davies, 1999; Parrot et al., 2003). There has been some criticism about whether microdialysis actually gets an accurate measure of normal brain levels of

metabolites since it is invasive (Timmerman and Westerink, 1998; Timmerman et al., 1999).

sut mice have brain atrophy in the striatum, cortical thinning and larger ventricles (Shih et al., 2006), however *xCT* mice do not show a reduction in cortical area (De Bundel et al., 2011). Perhaps the *xCT/sut* mice are more susceptible to perturbations of the brain than *xCT* mice due to the *sut* background. Introduction of a microdialysis probe could be more detrimental to *xCT/sut* mice and cause them to release more glutamate in response to introduction of the probe. One way to measure if *xCT/sut* mice did indeed have a reduction in extracellular glutamate in these brain areas would be to try and use a less invasive method than microdialysis to measure extracellular glutamate levels *in vivo*.

One such method is magnetic resonance spectroscopy (MRS), which could measure glutamate levels using radiolabelled substrates similar to magnetic resonance imaging (MRI) (Rothman et al., 2003).   The limitation to this technique is that it cannot distinguish between extracellular and intracellular space or vesicular compartments which would make it impossible to determine what the extracellular levels of glutamate are (Kegeles et al., 2012). Another possibility is the use of biosensors to measure glutamate levels (Wilson and Gifford, 2005).

Another possible way to determine if *SnJ* mice have more variable glutamate levels contributed by system xc- would be to measure the transporter's activity. *In vivo* glutamate levels could be measured through microdialysis by

infusing sodium free cystine and measuring glutamate output. This has been done in rats looking at cocaine addiction reinstatement and withdrawal, however they did not see an efflux of glutamate in normal rats that were not treated with cocaine (Baker et al., 2003a). The lack of change in normal rats could be due to the fact that they were measuring glutamate in the nucleus accumbens where this transporter might already be functioning maximally and therefore additional cystine will not cause more extrusion of glutamate. Other areas of the brain might have cystine dependent efflux of glutamate that could be a measure of transporter function, as has been seen in slices (Albano et al., 2013).

In vitro a synaptosome preparation can be used to measure transporter activity by measuring influx or efflux of radiolabelled cystine or glutamate (Nicholls et al., 1987; Breukel et al., 1997a; 1997b; Flynn and McBean, 2000). This preparation has been used to measure system xc- activity as well (Patel et al., 2004; Warren et al., 2004) and could be used with *SnJ* and *sut* (as a negative control) to see if the transporter functions similarly to *B6* mice in this preparation.

9.2.1 Other regulators of extracellular glutamate and their role in xCT

Another possible mechanism by which *sut* mice may have compensated for loss of system xc- could have been through downregulation of the excitatory amino acid transporters (EAATs). My data did not show that there were any differences in the amount of EAAT protein in any of the system xc- knockouts. This is consistent with data from Dr. Massie's lab that has shown no change in EAAT expression in the hippocampus of *xCT* knockouts (De Bundel et al., 2011).

However, in the current study EAAT levels were measured only in the whole brain and not specifically in the cerebellum and striatum where glutamate levels were measured. EAAT expression could be regulated in a very regional manner. In fact, it has been shown that the different EAATs are expressed in a region-specific manner (Gegelashvili and Schousboe, 1997; Furuta et al., 1997a; 1997b; Watase et al., 1998; Danbolt, 2001; Kanai and Hediger, 2004; Verrey et al., 2004; Dahlin et al., 2009; Kanai et al., 2013). It would be useful to see if EAAT expression is specifically changed in the striatum and cerebellum of the *sut* and *xCT/sut* system xc- knockout.

The antibodies used in the Western blotting were also only specific to one splice variant of each EAAT. Some of the EAATs have different splice variants that could be differentially expressed in these brain areas to control extracellular glutamate levels. EAAT-2 (Glt-1) has three splice variants that can have different roles during development (Reye et al., 2002a; 2002b; Yi et al., 2005). A more specific measurement of all of the splice variants may fine-tune whether EAAT expression is changed in system xc- mutants.

This study also failed to measure EAAT activity. Martinez et al showed that EAAT activity was down regulated after glutamate exposure, but there was no change in EAAT protein or mRNA (Martínez et al., 2013). EAAT activity could be downregulated in *xCT/sut* mice and would explain the lack of reduction in glutamate levels seen in these animals (Hayes et al., 2005). A study by Albano et al measured EAAT function in SOD1 knockout mice, a amytrophic lateral sclerosis model (ALS), and did not find a change in EAAT function but they did

see an increase in system xc- function (Albano et al., 2013). However, this study

was done in slice and did not measure EAAT function in system xc- mice (Albano

et al., 2013).

A measure of EAAT activity would be useful to determine the role of

EAATs in regulating extracellular glutamate levels and intracellular cystine

concentrations in system xc- mutants (Gegelashvili and Schousboe, 1997;

Danbolt, 2001). I did measure aspartate levels, which is a substrate for EAATs,

but did not see any change in these levels in any of the system xc- knockouts.

This could indicate that there is no change in EAAT function, however I did not

directly measure whether their glutamate uptake ability was changed in these

knockouts.

9.3 Conclusions

While this study did not show any consistent behavioral deficits across

system xc- mutant mice, there may be some subtle behavioral changes that can

be attributed to this transporter which have not been explored yet, such as sleep.

The relationship between system xc- and glutamate levels in the brain may be

region specific or variable in different background strains of mice. A

measurement of the activity of system xc- in the system xc- mice would be useful

to elucidate if the transporter functions similarly across different background

strains. There also could be compensatory changes in other regulators of

extracellular glutamate such as the EAATs that were too subtle to be detected

with Western blotting.

10 LITERATURE CITED

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Ye ZC, **Rothstein JD**, **Sontheimer H**. Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. *Journal of Neuroscience* 19: 10767–10777, 1999.

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11 APPENDIX

The following are figures that did not fit into the rest of the thesis. They
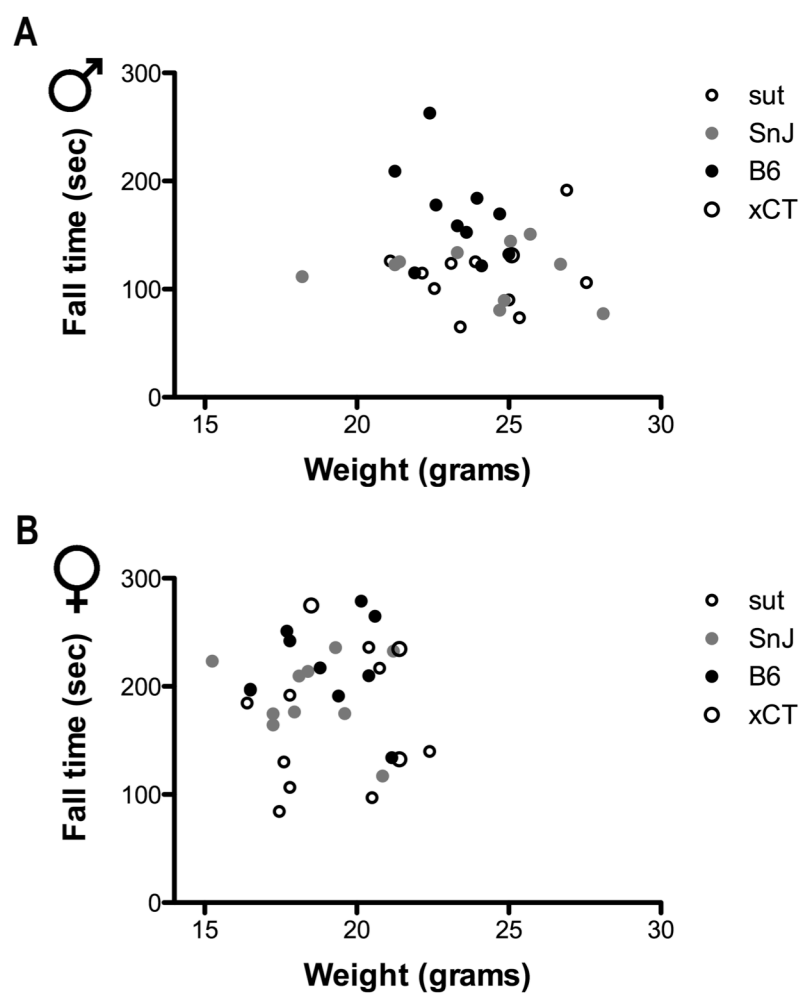
are supplemental to the previous chapters.

Figure 1, Rotarod weight.

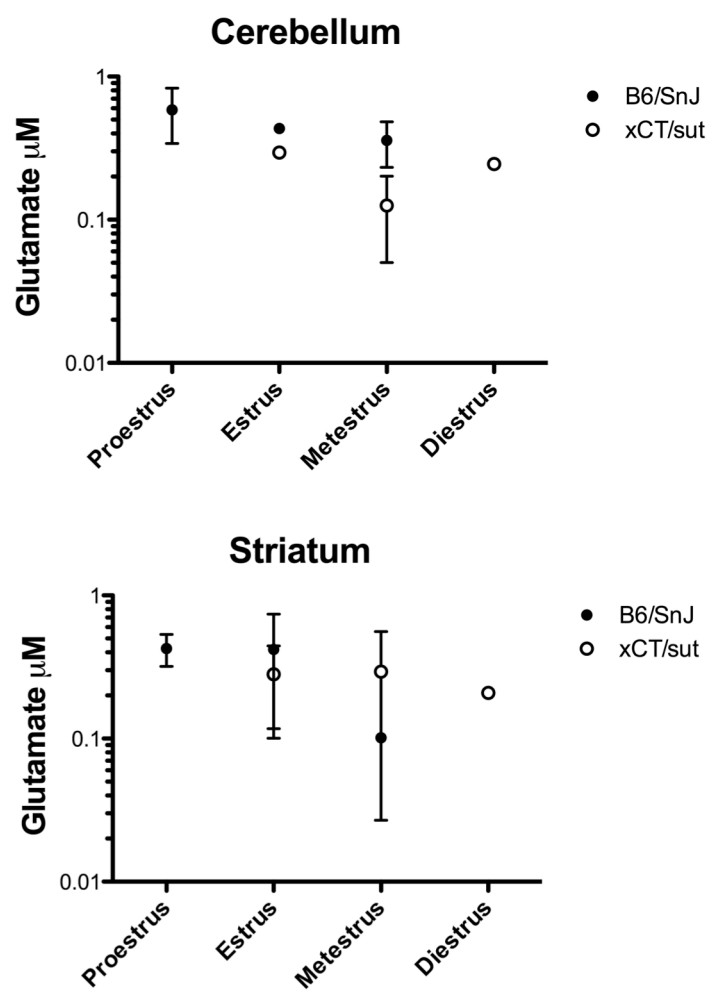Weight did not affect performance on the rotarod task for males (A) or females (B).

Cerebellum

Striatum

Figure 2 Estrus data for glutamate levels.

There was not enough data to quantify if glutamate levels change across the estrus cycle.

24 Hour Collection

3 Hour Collection

A

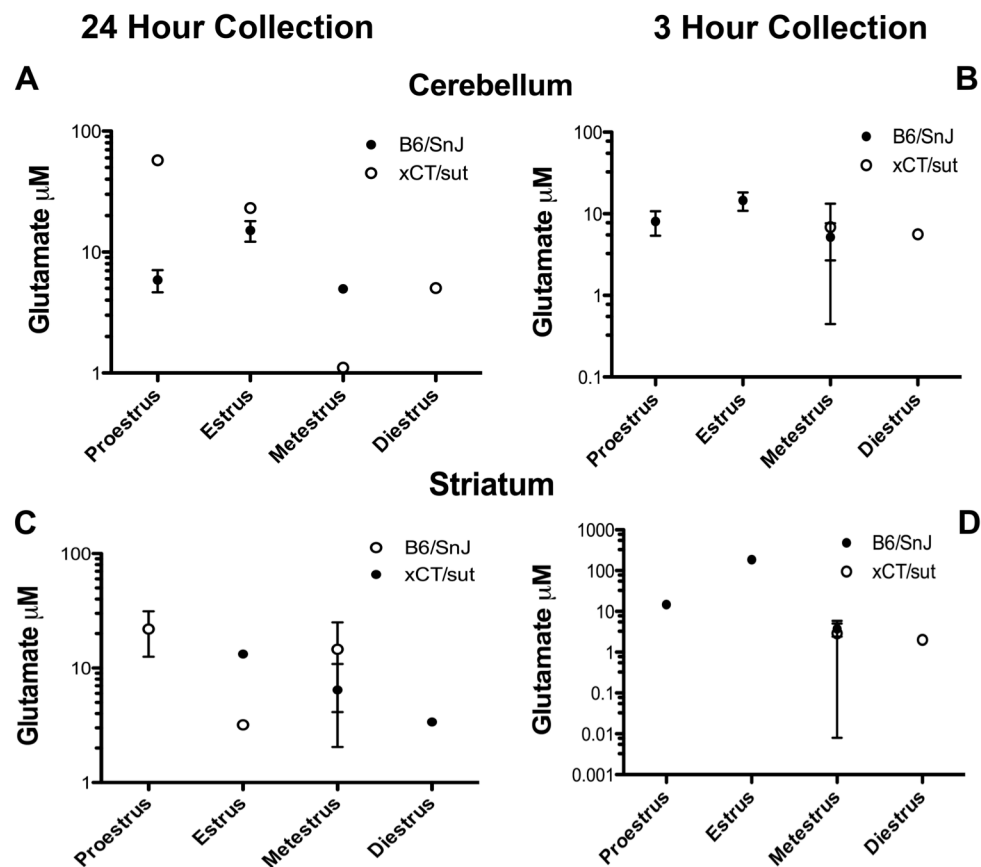**Cerebellum**

B

Striatum

C

D

Figure 3 Estrus data for slow flow glutamate levels.

There was not enough data to quantify if glutamate levels change across the estrus cycle.
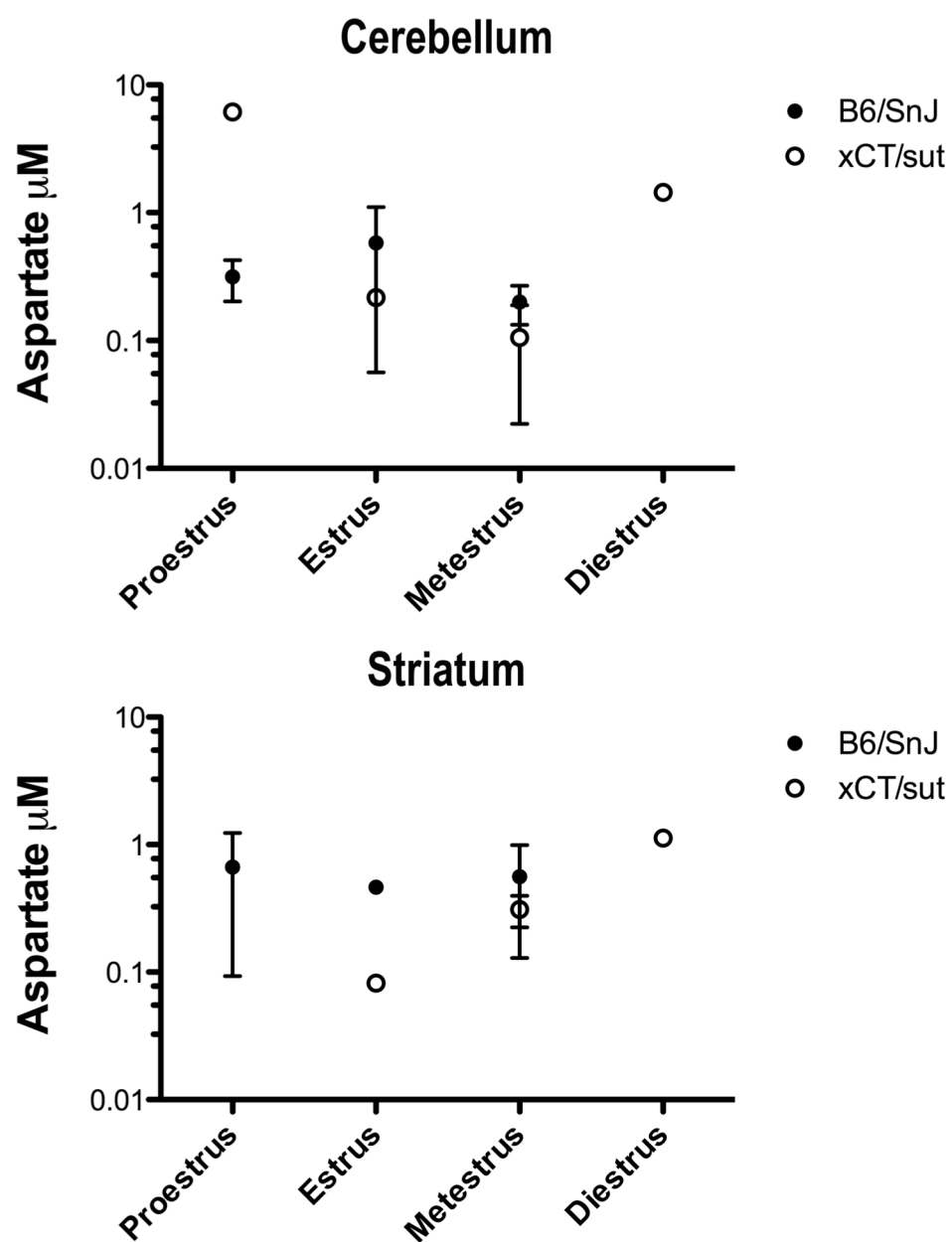
Figure 4 Estrus data for aspartate levels.

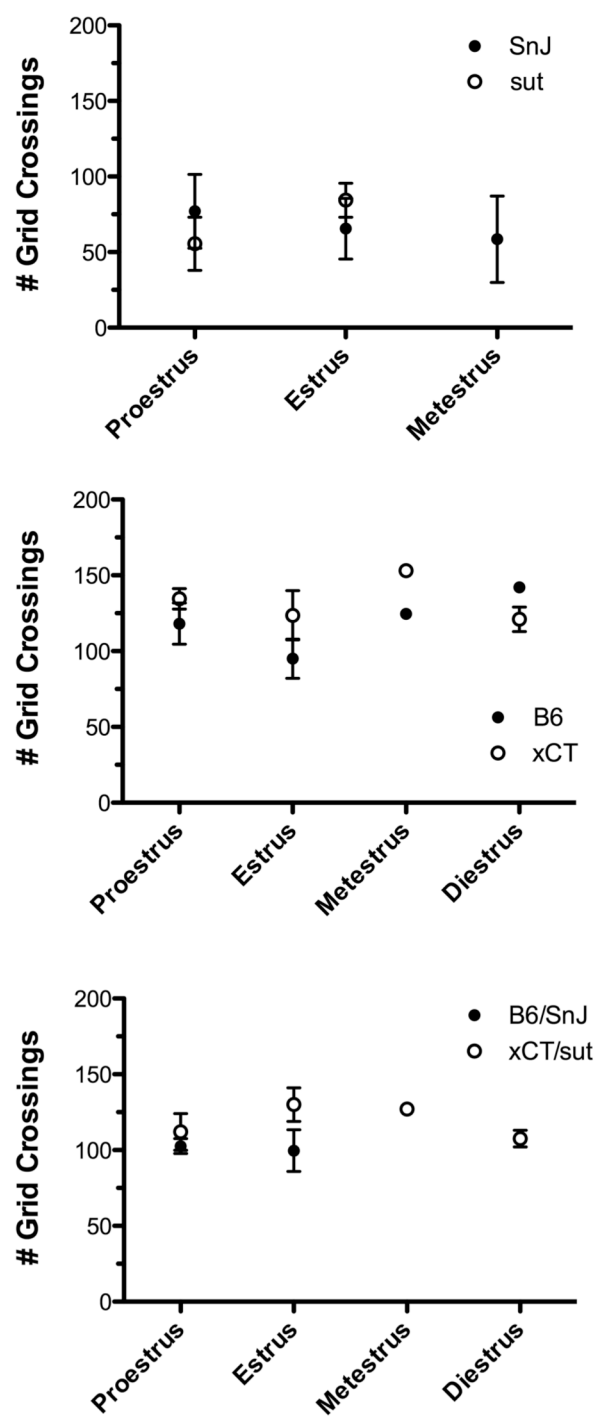There was not enough data to quantify if aspartate levels change across the estrus cycle.

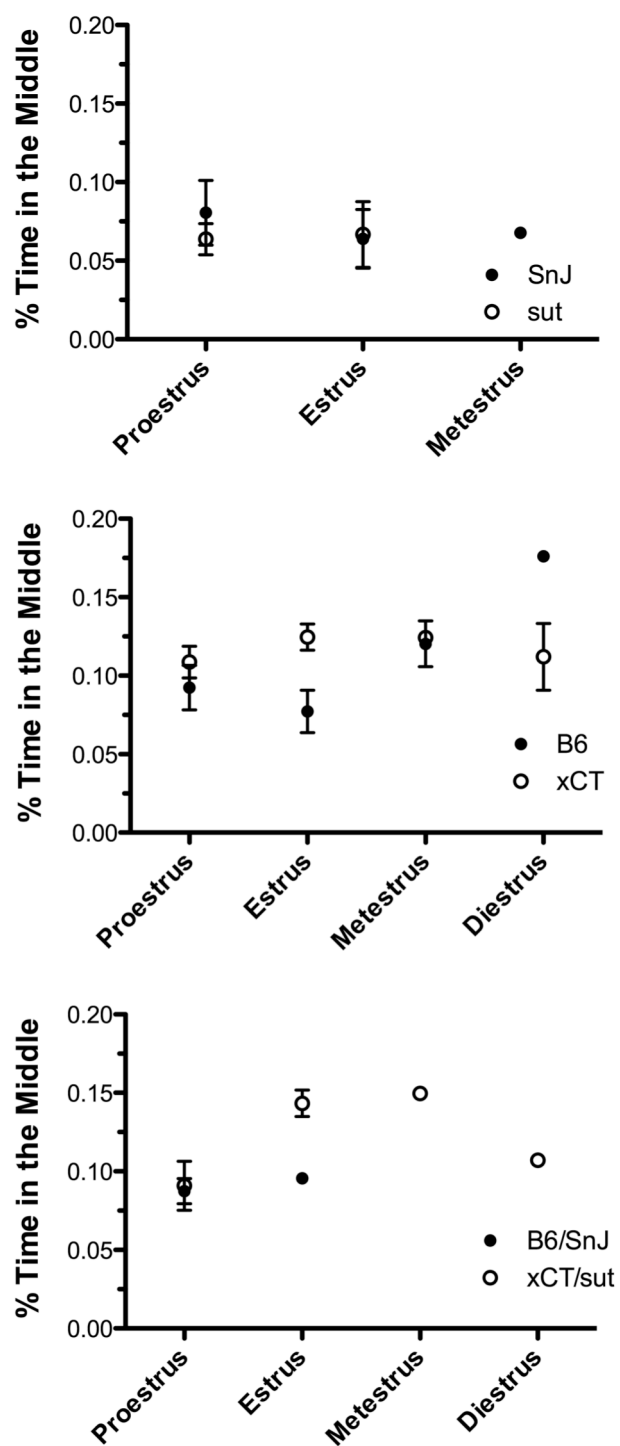**Figure 5 Open field number of grid crossings during the estrus cycle.**

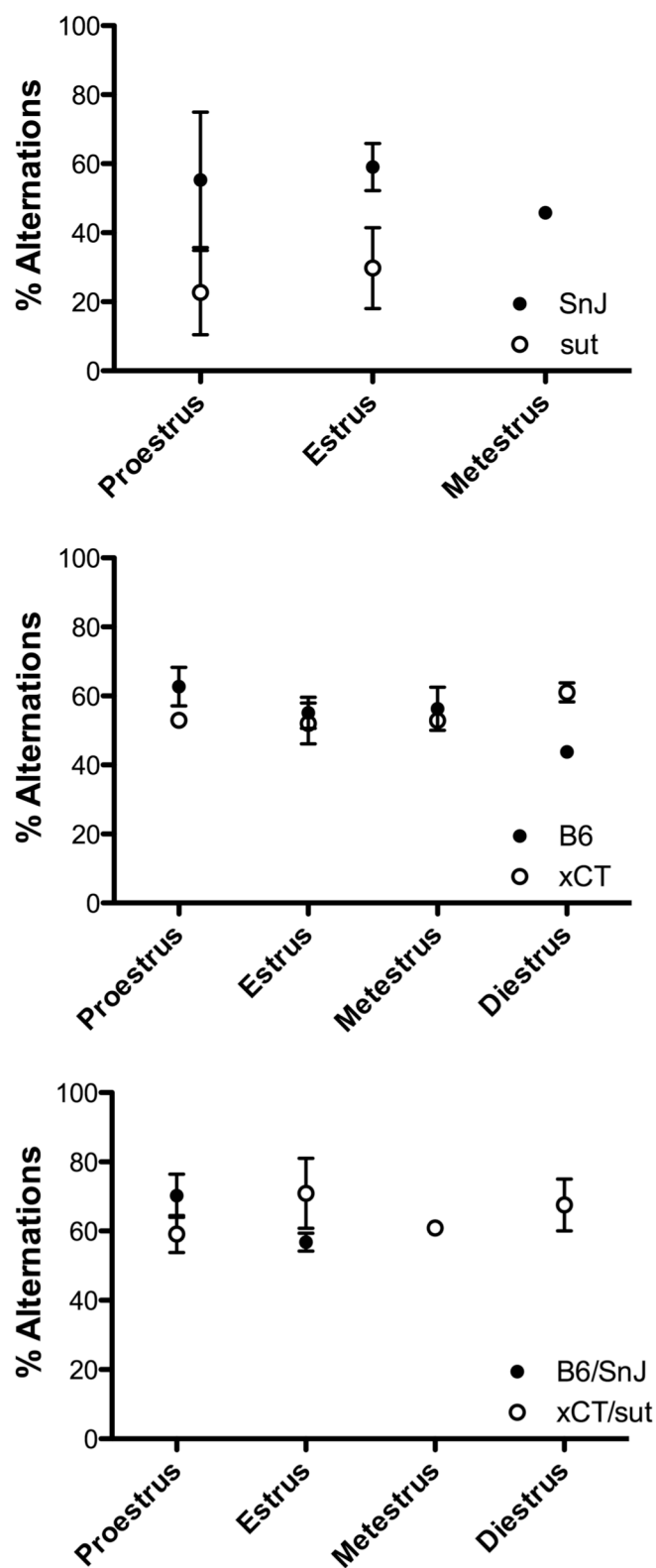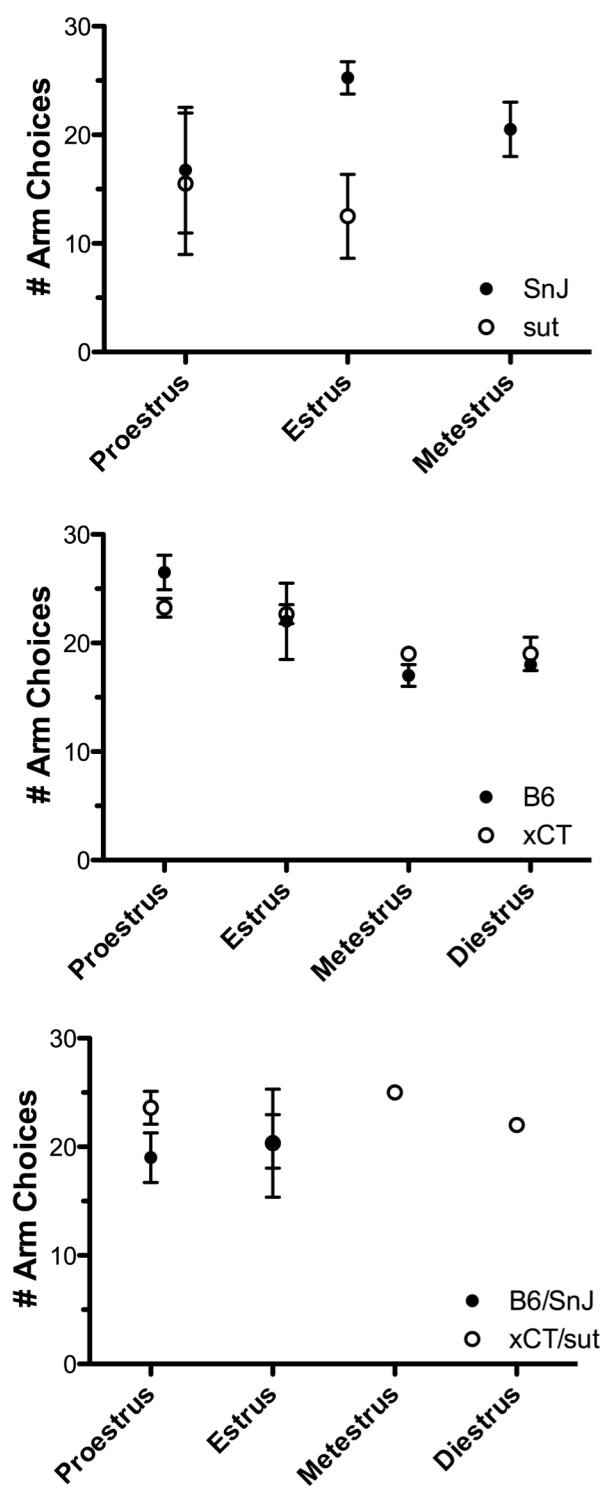**Figure 6 % time in the middle of the open field maze**

Figure 7 3 arm spontaneous alternation percent during the estrus cycle

Figure 8 3 arm number of choices during the estrus cycle

Elizabeth A Langer

Education:

B.S. Virginia Tech   Major: Biological Sciences/Psychology   2001-2005

M.S. Virginia Tech   Major: Biological Sciences   2005-2011:
Feather Mercury, Feather Color, and Fitness Effects in Eastern Bluebirds

PhD. University of Illinois Chicago Major: Biological Sciences   2008-current:
Behavioral Characterization of System xc- Mutants

Research Experience:

2004-2005 Field Study for Franklin Carvajal (Psychology dept.) learned scientific research methods including how to use the library. Developed methods for studying Post Traumatic Stress Disorder

Summer 2004, Field Study in Kenya, The School for Field Studies. Learned wildlife monitoring techniques, evaluated human-wildlife conflict near Amboseli National Park.

Summer 2005, Intern for Dr. John Phillips, research on sun compass in newts.

Summer 2006, 2007, Field research in Waynesboro, VA. Collected blood and feather samples from several species of birds, banded birds, monitored nest boxes, and kept accurate records.

2006-2007, measured spectral reflectance of Eastern Bluebird feathers

Sept 2008-Nov 2008 Rotation in Dr. Paul Malchow's lab evaluating Hydrogen ion concentration in catfish retinal horizontal cells

Nov 2008-current PhD research in Dr. David Featherstone's lab measuring behavioral effects of a glutamate-cystine transporter knockout in both Drosophila and mice.

Dec 2008-2011, collaboration with Dr. Janet Richmond on measuring behavioral changes in Tomosyn-RNAi Drosophila

Summer 2012, collaboration with Dr. Ann Massie to measure extracellular glutamate levels in *xCT* knockout mice using microdialysis

Grants

Spring 2006, GRDP, Virginia Tech, Amount
November 2010, WISE travel grant, UIC
November 2010, Biological Sciences Departmental Travel Grant, UIC
November 2010, Graduate Student Council Travel Grant, UIC
November 2010, Graduate College Travel Award, UIC
November 2011, Biological Sciences Departmental Travel Grant, UIC
November 2011, UIC Provost Award,
March 2012, LAS Travel Award
May 2012, Company of Biologists Travel Grant
May 2012, Boehringer Ingelheim Fond
August 2012, Graduate Student Council Travel Grant, UIC
October 2012, Biological Sciences Departmental Travel Grant, UIC

Publications

McCullagh E. A., and D.E. Featherstone Behavioral changes in system xc- mice, *Behavioral Brain Research,* in prep.

S. Borra, **E. A. McCullagh**, Featherstone, D.E., Baker, P., Ragozzino, M.E., and S.A. Shippy. Determining striatal extracellular glutamate levels in xCT mutant mice using LFPS CE-LIF, *Analyst*, submitted

Presentations

April 2009 Invited to present seminar on masters thesis work for the Ecology and Evolution Group in the Biological Sciences Department of University of Illinois Chicago.

Poster Presentation, **Langer, E.,** Park, T., Ragozzino, M., Featherstone D.E. Molecular control of decision-making involving cystine-glutamate transporters, Society for Neuroscience, Nov, 2010

Poster Presentation, **Langer, E.,** Park, T., Ragozzino, M., Featherstone D.E. Molecular control of decision-making involving cystine-glutamate transporters, Brain Research Foundation's Neuroscience Day, Dec 2010

Oral Presentation, Molecular control of decision-making involving cystine-glutamate transporters, UIC Graduate Student Symposium, February 2011,

Poster Presentation, **Langer, E.,** Park, T., Ragozzino, M., Featherstone D.E. Molecular control of decision-making involving cystine-glutamate transporters, CISAB Animal Behavior Conference at Indiana University, April 2011

Poster Presentation, **Langer, E.**, and D.E. Featherstone. Sulfasalazine injection does not affect behavior in a spontaneous alternation task through interaction with xCT, Society for Neuroscience, Nov, 2011

Poster Presentation, **Langer, E.**, and D.E. Featherstone. Behavioral Changes in Mice with Low Ambient Extracellular Glutamate Levels, Society for Experimental Biology, July 2012

Poster Presentation, **Langer, E.**, and D.E. Featherstone. Behavioral Changes in Mice with Low Ambient Extracellular Glutamate Levels, Society for Neuroscience, Oct. 2012

Teaching

Fall 2005, Instructor, Principles of Biology Laboratory, General Biology Lab
Spring 2006, Instructor, Principles of Biology Laboratory, General Biology Lab
Summer 2006, Upward Bound teaching Biology 2 to local high school students.
Fall 2006, Instructor, Principles of Biology Laboratory, General Biology Lab
Spring 2007, Instructor, Biological Principles Laboratory (Biology majors)
Fall 2008, Instructor, General Biology Laboratory
Spring 2009, Instructor, General Biology Laboratory
Summer 2009, Instructor, Microbiology Laboratory
Fall 2009, Instructor, Microbiology Laboratory
Spring 2010, Instructor, Microbiology Laboratory
Fall 2010, Instructor, Microbiology Laboratory
Spring 2011, Teaching Assistant, Neuroethology and Animal Behavior
Summer 2011, Teaching Assistant, Microbiology Lecture
Fall 2011, Instructor, Microbiology Laboratory
Spring 2012, Teaching Assistant, Neuroethology and Animal Behavior
Fall 2012, Teaching Assistant, Microbiology Laboratory
Spring 2013, Teaching Assistant, Microbiology Lecture
Summer 2013, Teaching Assistant, Microbiology Laboratory

Other Experience

Fall 2013 Office of Technology Management
-Screen new technologies
-Conduct market analysis and make decisions regarding new technologies
-Search for prior intellectual property to determine patentability of new technologies

Service

2005-2007, student member, Biology Department Diversity Committee

Fall 2006- distributed surveys to analyze diversity community among Graduate students
2006, Became member of Sigma Xi Honors Fraternity
Summer 2006- Panel for TA orientation regarding beginning teaching for new TAs
Fall 2006- assisted new non-English speaking instructor with teaching General Biology Labs
Fall 2007- helped organize Research in Progress Series (RIP) to encourage graduate students to present their research among their peers.
Spring 2011-current President of the Women's Ultimate Frisbee Club at UIC (jUICebox)
Tournament Director for qUICksilver Ultimate Frisbee Hat Tournament April 13th, 2013
2013, Member of the planning board for the inaugural Chicago Expanding Your Horizons Conference for girls 6-8 grade.

Media

2/19/11 'Knox Hosts 17th Annual Ultimate Frisbee Tourney' Galesburg Register Mail
http://www.galesburg.com/features/x449517055/Knox-hosts-17th-annual-Ultimate-Frisbee-tourney

7/27/11 'Zaarly's New Top Rockstar: Liz Langer' Zaarly Blog
http://blog.zaarly.com/blog/2011/07/27/zaarlys-new-top-rockstar-liz-langer/

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References

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