Regulation of the Cystine/Glutamate Antiporter and its Contribution to Neuronal Death

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REGULATION OF THE CYSTINE/GLUTAMATE ANTIPORTER AND ITS CONTRIBUTION TO NEURONAL DEATH

by

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ABSTRACT
REGULATION OF THE CYSTINE/GLUTAMATE ANTIPORTER AND ITS CONTRIBUTION TO NEURONAL DEATH

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The aim of this thesis is to better understand the regulation of the cystine/glutamate antiporter (system x_c-) and its role in regulating neuronal survival and death. Expressed primarily on astrocytes, system x_c- takes up cystine and releases glutamate in a 1:1 ratio. Cystine uptake is the rate-limiting step in glutathione synthesis, the brain's main antioxidant. Glutamate released into the extrasynaptic space can regulate neuronal function; however excessive glutamate release can cause excitotoxicity.

The dual actions of system x_c- make it of interest in many neurodegenerative diseases where oxidative stress and excitotoxicity are involved. We investigated the regulation of system x_c- in SOD1-G93A transgenic mouse model of ALS. We observed an increase in cystine uptake and glutamate release through system x_c- in spinal cord slices of SOD1-G93A transgenic mice. We did not observe a change in the function of the main glutamate clearance transporter, excitatory amino acid transporter (EAAT). This study was the first to show that system x_c- activity is dysregulated in an ALS model and suggests that the excitotoxicity in the SOD1-G93A transgenic mouse may be due to increased system x_c- activity.

Using primary mixed cortical cultures we assessed how different compounds that deplete intracellular glutathione (GSH), L-buthionine-sulfoximine (BSO) and diethyl maleate (DEM), affect system x_c- function. Both compounds caused significant decreases in intracellular GSH levels; however, DEM caused an increase in cystine uptake through system x_c-, while unexpectedly BSO caused a decrease in uptake. Also, DEM caused a decrease in intracellular cysteine, while BSO increased cysteine levels. The results suggest that negative feedback by intracellular cysteine is a more important regulator of system x_c- than intracellular GSH.

Transforming growth factor-β1 (TGF-β1) is a cytokine involved in regulating many cellular processes, including neuronal survival and death. We found that TGF-β1 increased cystine uptake through system x_c- in astrocyte-enriched glial cultures via the MAPK/ERK pathway. TGF-β1 increased the export of GSH from astrocytes, which suggests a neuroprotective role; however, in mixed cortical cultures TGF-β1 enhanced rotenone-induced neurotoxicity through AMPA receptors. The data suggests that the increase in system x_c- activity by TGF-β1 may have antioxidant defenses, but also exacerbates excitotoxicity.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AARE	amino acid response element
AD	Alzheimer's disease
ALK	activin receptor-like kinase
ALS	amyotrophic lateral sclerosis
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
APP	amyloid precursor protein
ARE	antioxidant responsive element
ATF4	activating transcription factor 4
ATP	adenosine tripohosphate
Aβ	amyloid-β
BSO	buthionine sulfoximine
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CHX	cycloheximide
CPG	S-4-carboxyphenyl glycine
CNS	central nervous system
CREB	cyclic-AMP response element binding proteis
CSF	cerebral spinal fluid
CysGly	cysteinylglycine
DAG	diacyl glycerol
DEM	diethyl methanol
DCF	dichlorofluorescein
EAAT	excitatory amino acid transporter
EAE	autoimmune encephalitis
eIF2	eukaryotic initiation factor 2
EpRE	electrophile response element
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FGF-2	fibroblast growth factor 2
GABA	γ-Aminobutyric acid
GCN2	general control nonderepressible protein 2
GLT-1	glial glutamate transporter -1
GS	glutathione synthase
GSH	glutathione
GSK3β	glycogen synthetase 3 β
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
HD	Huntington's disease
IGF-1	insulin-like growth factor 1
IL-1	interleukin 1-beta
IP_3	inositol 1,4,5-trisphosphate
K^+	potassium

LAP	latency-associated peptide
LDH	lactate dehydrogenase
LLC	large latent complex
LPS	lipopolysaccharide
LTBP	TGFβ binding protein
LTP	long-term potentiation
mACh	muscarinic aceylcholine
MAPK	mitogen-activated protein kinases
MCAO	middle cerebral artery occulsion
MCB	monochlorobimane
MEK	mitogen-activated protein kinase kinase
MEM	memantine
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
Mrp-1	multidrug resistance protein-1
MS	Multiple sclerosis
Na^{+}	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NASPM	1-naphthyl acetyl spermine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dion
NF-κB	nuclear factor-kappaB
NMDA	N-methyl-D-Aspartate
Nrf2	eythroid 2-related factor 2
$\bullet O_2$	superoxide
$\bullet OH^{-}$	hydroxyl radical
PACAP	Pituitary adenylate cyclase-activating polypeptid
PD	Parkinson's disease
PI_3	phosphatidylinositol 3
PI_3K	phosphatidylinositol 3 kinase
PLC	phospholipase C
PNC	pure neuronal culture
PIP2	phosphatidylinositol 4,5-bisphophate
PIP3	3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative real-time PCR
Slc7a11	solute carrier family 7, member 11
SOD-1	superoxide dismutase-1
SSZ	sulfasalazine
System x_c-	cystine/glutamate antiporter
TBOA	DL-threo-β-benzyloxyaspartic acid
tBOOH	tert-butyl hydroperoxide
TGF-β1	transforming growth factor – beta 1
TGF-βRI, -II	transforming growth factor – beta 1 receptor I and II

CHAPTER I

INTRODUCTION

General Introduction

The increase in human lifespan over the past several decades has been accompanied by an increase in the prevalence of neurodegenerative diseases. For example, in 2015 it was estimated that 5.3 million Americans had Alzheimer's disease (AD). Neurodegeneration is characterized by a loss of neuronal structure and function, including neuronal death. These diseases include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and many more. Although neurodegenerative diseases affect many people the cause of most of these diseases is still unknown. However, they share some common characteristics, including excitotoxicity and oxidative stress, which provides hope that a therapeutic treatment targeting these factors may be able to help in many of these diseases simultaneously.

The main focus of this thesis is to better understand the regulation of the cystine/glutamate antiporter (system x_c-) and the role it plays in regulating neuronal survival and death. Mainly located on astrocytes, system x_c- takes up cystine into the cell, where it is used to synthesize glutathione, the brain's main antioxidant (Figure 1.1). In exchange for taking up a molecule of cystine, system x_c- releases one molecule of glutamate. The glutamate released in the extrasynaptic space by system x_c- is able to regulate synaptic function. However, if glutamate levels become too high, they can also cause neuronal death through excitotoxicity. Therefore, the function of system x_c- allows it to play an important role in both oxidative stress and excitotoxicity and is likely to play

a role in neurodegenerative diseases.

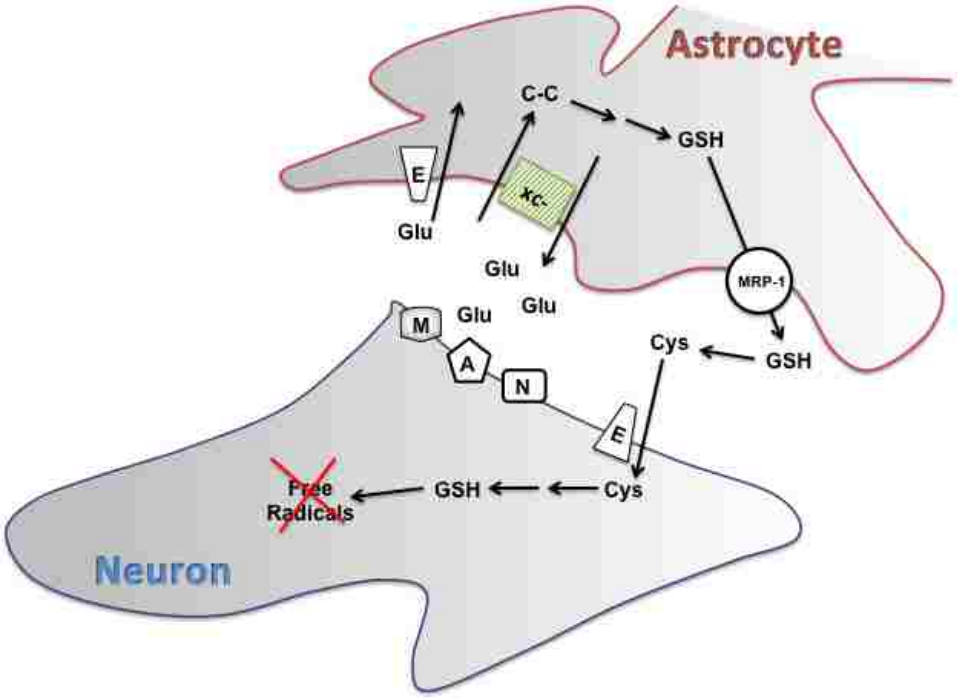

Figure 1.1 The function of system x_c^- in astrocytes and the effects it has on neurons. C-C: cystine; Cys: cysteine; GSH: glutathione; Glu: glutamate; xc-: system x_c^-; E: excitatory amino acid transporter; A: AMPA receptor; N: NMDA receptor; M: metabotropic glutamate receptor; MRP-1: multidrug resistance protein 1

GLUTAMATE NEUROTRANSMISSION

Glutamate is involved in most aspects of normal brain function. Glutamate is the primary excitatory neurotransmitter in the central nervous system (CNS); as such it is involved in brain development, cognition, memory, and learning (Erecińska and Silver, 1990; Meldrum, 2000). It is also important in many aspects of cellular metabolism, including stimulating glycolysis and protein synthesis (Dingledine et al., 1999) . Glutamate also has a wide array of indirect actions. It is a precursor to γ-aminobutyric acid (GABA) (Petroff, 2002), the main inhibitory neurotransmitter in the brain, and it is

one of the three main components of glutathione (GSH) (Meister and Anderson, 1983), the main endogenous antioxidant in the CNS.

Glutamate has multiple receptors through which it signals, including ionotropic and metabotropic receptors (Figure 1.2). Due to its many functions, glutamate levels are tightly regulated. Multiple transporters are responsible for clearing glutamate from the synapse in order to maintain proper glutamatergic signaling and prevent the overactivation of glutamate receptors, which can lead to cell death. Below is a brief review of some of these key aspects of glutamate neurotransmission.

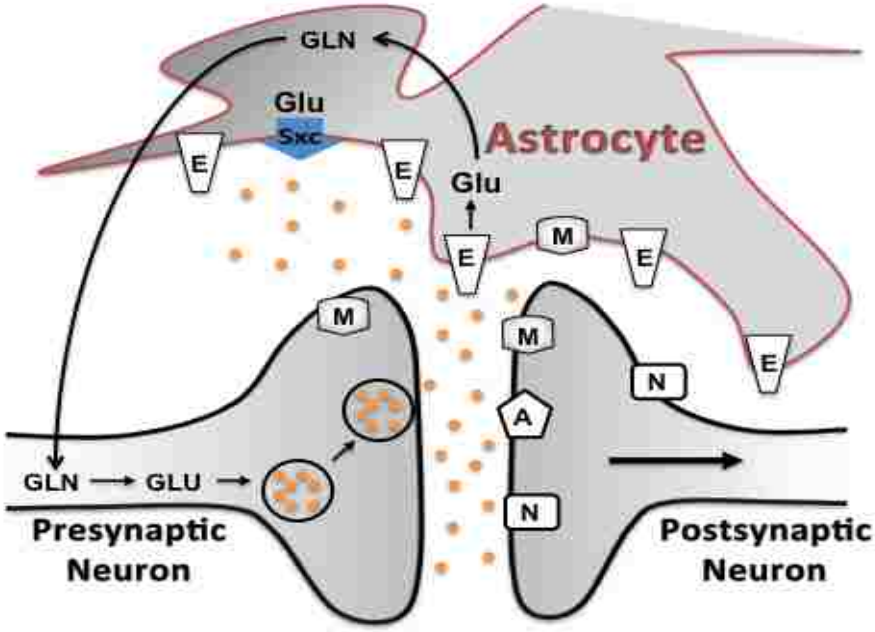

Figure 1.2 A typical tripartite glutamate synapse. A: AMPA receptor; N: NMDA receptor; M: metabotropic glutamate receptor receptor; E: excitatory amino acid transporter; Gln: glycine; Gln: glutamate; Sxc: system x_c-

Glutamate Receptors

There are two classes of glutamate receptors: ionotropic receptors and metabotropic receptors. Ionotropic receptors open to allow ions to flow in and out of the cell and are responsible for quick, short lasting responses. Metabotropic receptors, on the other hand, signal through an intracellular second messenger, which results in slower, longer lasting responses. Glutamatergic ionotropic receptors include 2-amino-3-(5-methyl-3- oxo-1,2- oxazol-4-yl)propanoic acid (AMPA)/kainate receptors and N-methyl-D-Aspartate (NMDA) receptors. There are eight metabotropic glutamate receptors (mGluR 1-8) that are divided into three main subgroups. Each type of receptor is briefly described.

AMPA/Kainate Receptors

AMPA receptors have a low affinity for glutamate (EC50 value: 200-500μM) (Meldrum, 2000) and are composed of a heterotetramer of four subunits (GluR1-4) (Meldrum, 2000; Traynelis et al., 2010). All AMPA receptors are permeable to sodium (Na^+) and potassium (K^+) and the permeability of the receptor to calcium (Ca^{2+}) is determined by the GluR2 subunit (Hollmann et al., 1991). Unlike the other subunits, GluR2 can undergo post-transcriptional editing (Sommer et al., 1990). Edited GluR2 subunits contain a positively charged arginine (R) residue instead of a neutral glutamine (Q) residue and are impermeable to Ca^{2+} (Sommer et al., 1991). Most AMPA receptors contain the edited GluR2 subunit and therefore are only permeable to Na^+ and K^+; GluR2-lacking AMPA receptors are very permeable to Ca^{2+} and activation can result in a rise in intracellular Ca^{2+} levels (Isaac et al., 2007; Traynelis et al., 2010).

AMPA receptors are not voltage dependent and open and close quickly. They mediate the fast initial part of the excitatory postsynaptic potential (EPSP) and are responsible for most of the fast excitatory synaptic transmission in the brain (Zhang and Trussell, 1994; Edmonds et al., 1995). AMPA receptor trafficking into and out of the postsynaptic membrane plays a key role in determining the strength of the synapse (Malinow and Malenka, 2002; Shepherd and Huganir, 2007; Anggono and Huganir, 2012).

Kainate receptors are composed of a tetramer of four subunits (GluK1-5). They are permeable to Na^+ and K^+ and are located on both postsynaptic and presynaptic neurons (Chittajallu et al., 1999; Dingledine et al., 1999; Meldrum 2000). The function of kainate receptors is not as well defined. However, it is known that they act as modulators of synaptic activity through a variety of mechanisms, including regulating the release of both glutamate and GABA from presynaptic neurons and regulating the excitability of postsynaptic neurons (Contractor et al., 2011).

NMDA Receptors

NMDA receptors have a high affinity for glutamate (EC50: 2.5-3μM) (Meldrum, 2000) They are a heterotetramer composed of two GluN1 and two GluN2 (GluN2A-D) or GluN3 subunits; the distribution of isoforms are regionally and developmentally specific (Watanabe et al., 1992; Laube et al. 1998; Dingledine et al., 1999). There are two main ways in which NMDA receptors are distinct from AMPA receptors. First, under physiological conditions NMDA receptor channels are blocked by magnesium (Mg^{2+}) (Nowak et al., 1984; Dingledine et al., 1999). In order for the Mg^{2+} block to be removed, the cell membrane must first be depolarized by AMPA receptors. Once the Mg^{2+} block is

removed, Na^+, K^+, and Ca^{2+} can flow through the NMDA receptor. Second, NMDA receptor activation requires the binding of the co-agonist glycine or D-serine (Wilcox et al., 1996; Laube et al. 1998). Glutamate/NMDA binds to the GluN2 subunit and glycine/D-serine binds to the GluN1 subunit. NMDA receptors also have multiple allosteric modulation sites, which affect the activity of the channel (Dingledine et al., 1999).

Due to its slower kinetics the NMDA receptor is responsible for producing the slower, longer lasting part of the EPSP (Vargas-Caballero and Robinson, 2004). NMDA receptor activation leads to a rise in intracellular Ca^{2+} concentrations, which can activate many signaling pathways and is critical for synaptic plasticity, a mechanism of learning and memory (Malenka and Nicoll, 1993; Benfenati, 2007; Lüscher and Malenka, 2012).

Metabotropic Glutamate Receptors

Metabotropic receptors consist of an extracellular N-terminus that contains a ligand-binding site, seven transmembrane domains, and an intracellular C-terminus that couples to a G-protein (Conn and Pin, 1997; Niswender and Conn, 2010). Activating metabotropic receptors leads to the activation of the G-coupled protein and an intracellular signaling cascade. There are eight metabotropic glutamate receptors (mGluRs) subdivided into three functional groups (Conn and Pin, 1997; Niswender and Conn, 2010; Willard and Koochekpour, 2013). Group I consists of mGluR1 and mGluR5 and are coupled to G_q proteins (Hermans and Challiss, 2001; Ribeiro et al., 2010). G_q proteins primarily activate phospholipase C (PLC), which cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacyl-glycerol (DAG). IP_3 releases Ca^{2+} from the endoplasmic reticulum and DAG stimulates protein kinase C

(PKC). Both Group II (mGluR2 and mGluR3) and Group III (mGluR4-8) metabotropic glutamate receptors are coupled to G_i proteins (Conn and Pin, 1997; Niswender and Conn, 2010; Willard and Koochekpour, 2013). Activation of G_i proteins primarily inhibits adenylyl cyclase (AC), which decreases cyclic adenosine monophosphate (cAMP).

mGluRs are primarily located perisynaptically and extrasynaptically on both postsynaptic and presynaptic neurons and modulate synaptic activity (Conn, 2003). Activation of postsynaptic mGluRs has been shown to modulate the activity of ion channels and presynaptic mGluRs inhibit the release of neurotransmitters (Conn and Pin, 1997; Conn, 2003; Ribeiro et al., 2010). mGluRs are also located on glia and their activation can lead to the release of glutamate from astrocytes and regulate the expression and function of glutamate transporters (D'Antoni et al., 2008). Glutamate binds to each of the receptors with varying affinity (Meldrum, 2000).

Glutamate Clearance

Clearing glutamate from the synapse is very important for terminating excitatory transmission and for keeping extracellular concentration of glutamate below excitotoxic levels (Nicholls and Attwell, 1990; Kanai and Hediger, 1992; Rothstein et al., 1996; Seal and Amara, 1999; Liang et al., 2008). Excitatory amino acid transporters (EAATs) are high capacity glutamate transporters and are the primary mechanism responsible for the uptake of glutamate out of the synapse (Kanai and Hediger, 1992; Seal and Amara, 1999; Bridges et al., 2012a). Intracellular glutamate concentrations are in the millimolar range, while extracellular concentrations are kept in the low micromolar range (Ronne-Engström et al., 1995; Baker et al., 2003). In order to create such a steep glutamate

concentration gradient, the uptake of one molecule of glutamate is coupled to the uptake of three molecules of Na^+ and one molecule of hydrogen (H^+) and the efflux of one molecule of K^+ (Zerangue and Kavanaugh, 1996a). Five EAATs (EAAT 1-5) have been cloned. EAAT1/GLAST and EAAT2/GLT-1 are located on astrocytes and are responsible for the majority of glutamate clearance from the synapse (Rothstein et al., 1994; Seal and Amara, 1999). EAAT3 and EAAT4 are located on neurons and EAAT5 is primarily expressed in the retina (Rothstein et al., 1994; Wersinger et al., 2006).

Glutamate Microdomains

Along with clearing glutamate from the synapse in order to terminate excitatory signaling, EAATs located on astrocytes can prevent glutamate in one synapse from diffusing into and activating receptors in neighboring synapses (Rusakov, 2001). Similarly, EAATs can also prevent glutamate released into the extrasynaptic space by astrocytes from diffusing into the synaptic zone (Jabaudon et al., 1999). In this way, EAATs create microdomains of glutamate (Bridges et al., 2012a). In the hippocampal synapses glutamate levels peak around 1.1mM, but quickly decay (Clements et al., 1992). Basal glutamate levels in the extrasynaptic space are 1 to 3μM (Baker et al., 2002), which is high enough to activate some types of extrasynaptic receptors.

Activation of synaptic and extrasynaptic glutamate receptors by these separate microdomains of glutamate can lead to different functional outcomes. One of the most well studied examples is the NMDA receptor. Its activation is critical for neuronal survival, but at the same time activation can also lead to cell death. Evidence suggests that this 'NMDA paradox' is a result of the receptor's location (Hardingham and Bading, 2010). It is generally accepted that stimulating synaptic NMDA receptors activate

intracellular pathways that aid in neuroprotection, while stimulation of extrasynaptic NMDA receptors activate pathways that promote cell death (Hardingham et al., 2002; Léveillé et al., 2008). When synaptic NMDA receptors are stimulated the influx of calcium into the cell induces cyclic-AMP response element binding protein (CREB) (Hardingham et al., 2002), a transcription factor that regulates many cell survival genes and has a well-documented role in neuronal protection (Bonni et al., 1999; Mayr and Montminy, 2001). Synaptic NMDA receptor activation also inhibits the apoptotic cascade and protects against oxidative stress (Hardingham and Bading, 2010; Léveillé et al., 2010; Papadia et al., 2008). On the other hand, stimulating extrasynaptic NMDA receptors turn off CREB activity and leads to mitochondrial dysfunction (Hardingham et al., 2002). Extrasynaptic NMDA receptor activation also inhibits extracellular signal-regulated kinase (ERK) (Ivanov et al., 2006; Léveillé et al., 2008), a kinase that has been shown to play a role in synaptic NMDA receptor neuroprotection (Hetman and Kharebava, 2006).

Excitotoxicity

While glutamate is necessary for normal brain function, just a brief exposure of mature cortical neurons to high levels of glutamate can cause morphological changes and neuronal death occurs within a few hours (Choi et al., 1987). Under normal physiological conditions extracellular glutamate concentrations are tightly regulated, but if the regulation is disrupted and extracellular glutamate concentrations rise, excitotoxicity can occur. Excitotoxicity is the damage and death of neurons, caused by an over activation of glutamate receptors (Choi et al., 1987). It is a main pathology in many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS),

Alzheimer's disease (AD), multiple sclerosis (MS), and Parkinson's Disease (PD) (Leigh and Meldrum, 1996; Doble, 1999; Koutsilieri and Riederer, 2007; Dong et al., 2009). It is also a key pathology in stroke/ischemia and traumatic brain injury (Choi, 1988; Hayes et al., 1988; Yi and Hazell, 2006; Szydlowska and Tymianski, 2010).

Excitotoxicity is calcium dependent (Choi, 1985, 1987; Tymianski et al., 1993). Overactivation of glutamate receptors results in a large influx of calcium into the cell, which activates multiple enzymes. Activation of these enzymes leads to an increase in free radicals, protein breakdown, cell membrane degradation, and DNA damage, culminating in cell death (Freeman and Crapo, 1982; Mark et al., 2001; Berliocchi et al., 2005). Excitotoxicity can occur as a result of NMDA receptor overactivation. For example, *in vitro* NMDA receptor antagonists significantly block cell death induced by glutamate (Choi et al., 1988; Tymianski et al., 1993). NMDA overactivation is also involved in many models of disease. Microglia treated with amyloid-beta (Aβ), a hallmark of AD, have increased glutamate release, which results in enhanced cortical neuron toxicity. Multiple NMDA receptor antagonists can block this enhanced toxicity (Qin et al., 2006). *In vivo*, excitotoxicity caused by Aβ is blocked by the NMDA receptor antagonist, memantine (MEM) (Miguel-Hidalgo et al., 2002). In clinical trials, MEM has been shown to delay clinical signs of AD (Hellweg et al., 2012). It has also been shown to be protective in other models of excitotoxicity, including the 1-methyl-4-phenylpyridinium (MPP+) toxicity model of Parkinson's disease and an *in vivo* model of hypoxia-ischemia (Volbracht et al., 2006), suggesting that NMDA receptor overactivation is involved.

Excitotoxicity can also occur as a result of overactivation of GluR2-lacking AMPA receptors. Motor neurons are vulnerable to kainate toxicity because they have a high number of GluR2-lacking AMPA receptors; and calcium entry through these receptors is responsible for the toxicity (Carriedo et al., 1996; Van Den Bosch et al., 2000; Van Damme et al., 2002). In presymptomatic SOD1-G93A mouse, a model of ALS, an increase in GluR2-lacking AMPA receptors has been observed and AMPA receptor antagonists have been shown to increase motor neuron survival and prolong the survival of these mice (Van Damme et al., 2003; Tortarolo et al., 2006). Overactivation of AMPA receptors may also play a role in MS. In oligodendrocyte cultures, kainate or increasing extracellular glutamate levels leads to excitotoxicity, which can be blocked by AMPA receptor antagonists (Yoshioka et al., 1996; Domercq et al., 2005). AMPA receptor antagonists have been shown to also improve neurological scores in mice treated with autoimmune encephalitis (EAE), a model of MS (Smith et al., 2000).

NEURON-ASTROCYTE INTERACTION

Astrocytes, the most common type of glial cell in the brain, are highly organized. They ensheath synapses and have non-overlapping domains (Ventura and Harris, 1999; Bushong et al., 2002; Nedergaard et al., 2003). Traditionally, astrocytes are thought of as playing a supportive role to neurons; more recently, however, many studies are emerging that show astrocytes are a key player in regulating neurotransmission. Below is a brief review of some important aspects of the neuron-astrocyte interaction.

Metabolic Coupling and the Glutamate/Glutamine Cycle

Glucose is the main substrate for energy production in the brain and the astrocyte-neuron interaction is critical for maintaining energy homeostasis, as well as normal glutamatergic transmission. After glutamate is released into the synapse it is taken up by astrocytes through EAATs, along with three molecules of Na^+ (Zerangue and Kavanaugh, 1996a). The increase in intracellular Na^+ concentration activates the Na^+/K^+ pump, which triggers astrocytes to take up glucose from capillaries, which are covered by astrocytic end feet. The activation of the Na^+/K^+ pump also triggers glycolysis, which metabolizes glucose into lactate, a process that yields two molecules of adenosine triphosphate (ATP) (Pellerin and Magistretti, 1994; Magistretti and Pellerin, 1999). The lactate is then released by astrocytes and taken up by neurons, where it is converted into pyruvate and goes through the tricarboxylic acid (TCA) cycle to supply the neuron with energy (Tsacopoulos and Magistretti, 1996; Magistretti and Pellerin, 1999). The two molecules of ATP produced by glycolysis are used to convert glutamate to glutamine by the enzyme glutamine synthase. Glutamine is then released and taken up by neurons, where it is converted back into glutamate (Sibson et al., 1998). This metabolic interaction is illustrated in Figure 1.3. Since this cycle depends on uptake of glutamate from the synapse following neurotransmission, EAAT activation is an activity-dependent signaling mechanism for the utilization of glucose (Sibson et al., 1998; Magistretti and Pellerin, 1999).

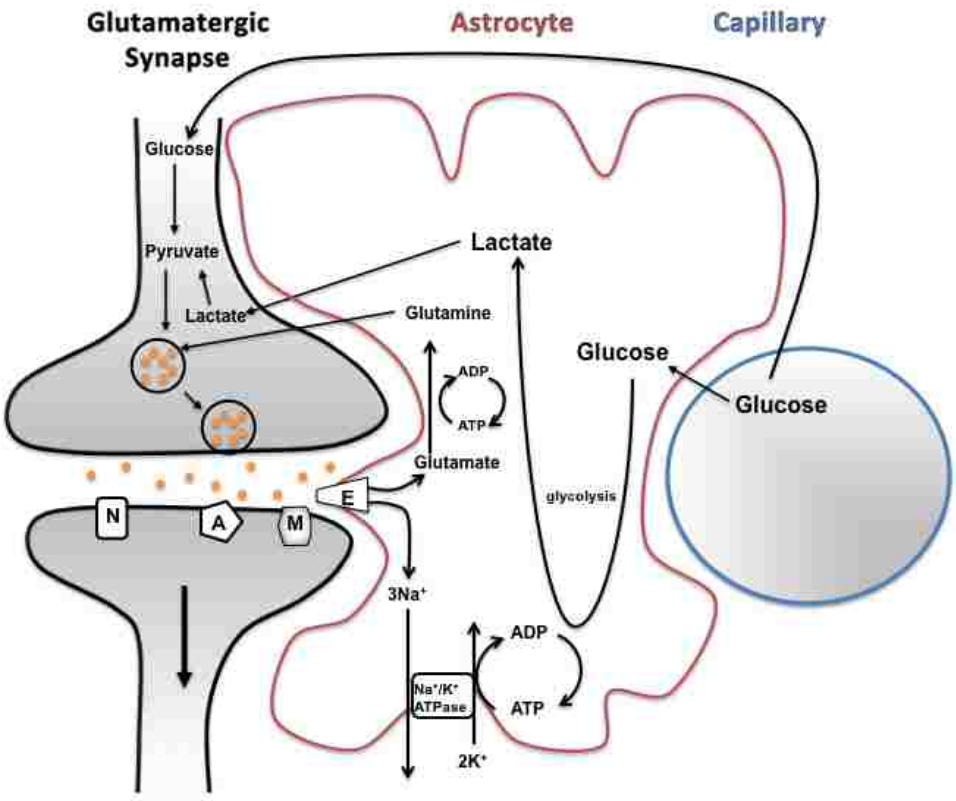

Figure 1.3 Interaction between astrocytes and neurons: metabolic coupling and the glutamate/glutamine cycle. A: AMPA receptor; N: NMDA receptor; M: metabotropic glutamate receptor receptor; E: excitatory amino acid transporter Adapted and modified from (Magistretti and Pellerin, 1999)

Neuronal Regulation of Astrocytes

Like neurons, astrocytes express a wide variety of ion channels and neurotransmitter receptors, including K^+ channels, ionotropic glutamate receptors, mGluRs, GABA receptors, muscarinic aceylcholine (mAch) receptors and endocannabinoid (CB) receptors (Barres, 1991; Newman, 2003; Navarrete and Araque, 2008). Neuronal activity can activate these receptors and lead to a wide range of effects. For example, astrocyte membranes can be depolarized in response to glutamate or in response to high concentrations of extracellular K^+ that result from high neuronal activity

(Bowman and Kimelberg, 1984; Sontheimer et al., 1988; Usowicz et al., 1989; Murphy et al., 1993; Meeks and Mennerick, 2007). GABA released from neurons has been shown to regulate astrocyte morphology through $GABA_A$ receptors (Matsutani and Yamamoto, 1997) . Although the mechanism is unknown, neurons can also regulate expression of glutamate transporter subtypes (Swanson et al., 1997). Activation of many of the G-protein coupled receptors on astrocytes leads to increased intracellular concentrations of Ca^{2+} (Porter and McCarthy, 1996; Araque et al., 2002; Newman, 2003; Navarrete and Araque, 2008).

Astrocyte Regulation of Neurons

Astrocytes are involved in most aspects of synaptic transmission (Araque et al., 1999; Newman, 2003; Bridges et al., 2012a). They take part in synaptogenesis by influencing synapse formation, axonal pruning, and synapse elimination through secreted and cell-surface signals (Ullian et al., 2001; Eroglu and Barres, 2010). Intracellular Ca^{2+} waves can cause astrocytes to release vesicular gliotransmitters that regulate neuronal function and synaptic plasticity (Araque et al., 1999, 2000; Newman, 2003). For example, glutamate release from astrocytes can increase neuronal Ca^{2+} levels (Parpura et al., 1994). Astrocytes influence synaptic plasticity by enhancing neuronal NMDA receptor activation and enabling induction of long-term potentiation (LTP) through release of D-serine (Yang et al., 2003) . Non-vesicular release of glutamate from astrocytes through the cystine/glutamate antiporter (system x_c-) can also influence neuronal transmission through activation of extrasynaptic glutamate receptors (Bridges et al., 2012a; 2012b; Massie et al., 2015).

Due to its highly branched morphology, one astrocyte is able to interact with tens of thousands of synapses (Halassa et al., 2007), which allows them to synchronize neuronal activity (Rouach et al., 2008). It also allows for neurons to influence synaptic transmission of neighboring synapses by signaling through astrocytes (Araque et al., 1999; Angulo et al., 2004; Navarrette and Araque, 2008). And although astrocytes have non-overlapping domains, they are coupled together by gap junctions (Dermietzel et al., 1991; Giaume et al., 1991), through which signals spread rapidly between astrocytes, allowing them to work together as a network (Rouach et al., 2008; Giaume et al., 2010).

OXIDATIVE STRESS

A common pathology in many neurodegenerative diseases discussed in this thesis is oxidative stress. Oxidative stress is an accumulation of reactive oxygen species (ROS) due to an imbalance between the production of ROS and the body's ability to eliminate them. Common ROS include superoxide ($\bullet O_2^-$), hydroxyl radical ($\bullet OH^-$) and hydrogen peroxide (H_2O_2). The brain is particularly vulnerable to oxidative stress because of its high oxygen consumption.

Production and Elimination of ROS

There are two main sources of intracellular ROS production. First, ROS are produced during normal mitochondrial oxidative metabolism. During cellular respiration electrons from the mitochondria leak out and react with oxygen, forming $\bullet O_2^-$, the precursor of most other ROS (Freeman and Crapo, 1982; Kirkinezos and Moraes, 2001; Turrens, 2003) . The second source is from activity of the membrane bound enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidases are

found in neurons, astrocytes, and microglia; and when active they generate $\bullet O_2^-$ as electrons cross the cell membrane and couple with oxygen (Infanger et al., 2006; Sorce and Krause, 2009) .

As seen in Figure 1.4 cells have various defense mechanisms against ROS, including superoxide dismutases (SOD), catalase, and glutathione (GSH). SODs are a class of enzymes that catalyze $\bullet O_2^-$ into H_2O_2 (Fridovich, 1975). While H_2O_2 itself is not reactive, the bond between the two oxygen atoms is very weak and it can form the highly reactive hydroxyl radical $\bullet OH^-$ via the Fenton reaction (Winterbourn, 1995). Some H_2O_2 reacts with a catalase to form the non-harmful byproducts, water and oxygen (George, 1947). The majority of H_2O_2 is removed by GSH in a reaction catalyzed by glutathione peroxidase, which oxidizes GSH into glutathione disulfide (GSSG) and forms water (Finley et al., 1981; Winterbourn and Metodiewa, 1994). GSSG can be converted back into GSH by glutathione reductase and the reduced form of NADPH (Simonian and Coyle, 1996). Under normal conditions, 99% of total cellular GSH is in the reduced form. However, when oxidative stress occurs, GSSG is rapidly produced and accumulates in the cell (Deneke and Fanburg, 1989).

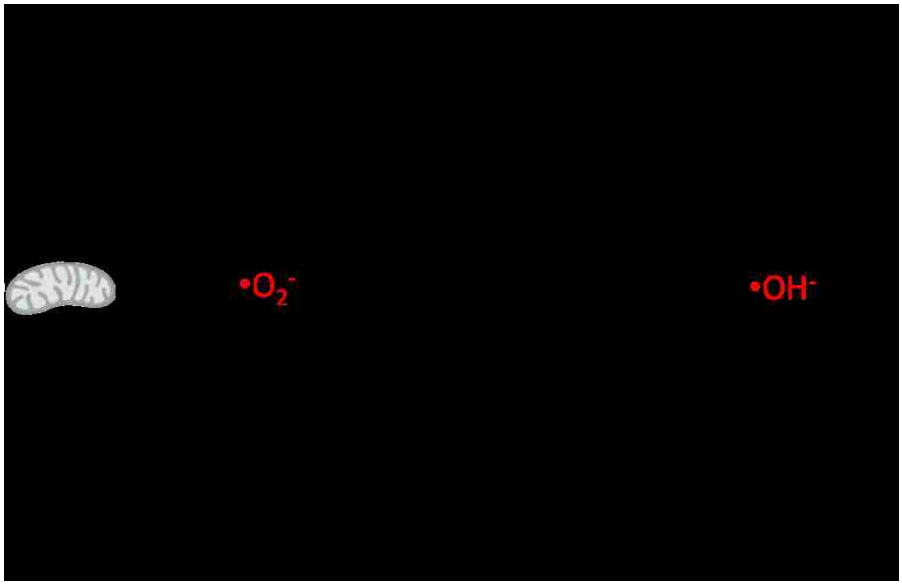

Figure 1.4 Cellular pathways involved in regulating •O_2. GSH: glutathione; GSSG: oxidized glutathione; H_2O: water; O_2: oxygen: O_2-: superoxide; OH-: hydroxal radical; H_2O_2: hydrogen peroxide; Fe: iron

Oxidative stress occurs when there is an accumulation of ROS, due to an imbalance between ROS production and removal, which can occur through a number of mechanisms. For example, middle cerebral artery occlusion in mice upregulates NADPH oxidase expression in the mouse brain resulting in an increase in ROS production (Vallet et al., 2005) . Mitochondrial dysfunction is a common mechanism behind increased ROS production; multiple new therapeutics are aimed at targeting the mitochondria because mitochondrial dysfunction is a main pathology in many neurodegenerative diseases (Lin and Beal, 2006; Federico et al., 2012; Lee, 2016). Decreased ROS removal can also make cells more susceptible to oxidative damage. For example, dopaminergic neurons co-cultured with astrocytes show increased toxicity to H_20_2 when the astrocytes have been depleted of GSH (Drukarch et al., 1997). Increased intracellular ROS causes damage to DNA, protein oxidation, and damage to the plasma membrane via lipid

peroxidation (Freeman and Crapo, 1982; Berliocchi et al., 2005). Prolonged exposure to oxidative stress leads to cell death.

Glutathione Metabolism

GSH, a tripeptide composed of cysteine, glutamate, and glycine, is the major antioxidant found in the brain. GSH synthesis occurs intraceullularly in a two-step process (Meister and Anderson, 1983). First, the enzyme glutamate-cysteine ligase (GCL) catalyzes glutamate and cysteine to produce the dipeptide, γ-glutamylcysteine (γGluCys). Glycine is then added to the dipeptide by GSH synthase (GS) to form GSH. Both steps require ATP and GSH metabolism is dependent upon the availability of glutamate, cysteine, and glycine. Since glutamate and glycine are highly available, the uptake of cystine or cysteine into the cell is the rate-limiting step in GSH synthesis (Sagara et al., 1993a; Dringen and Hirrlinger, 2003).

There are two well characterized mechanisms found on astrocytes and neurons that take up cystine or cysteine: system x_c- and the glutamate/aspartate/cysteine transporter (EAAC1/EAAT3). Astrocytes transport cystine, mainly through system x_c- (Sagara et al., 1993a; Kranich et al., 1998; Bridges et al., 2012b). Once inside the cell, cystine is rapidly reduced into two cysteine molecules and metabolized into GSH (Dringen and Hirrlinger, 2003). Neurons, on the other hand, have limited ability to take up cystine as a GSH precursor. Instead neurons rely on cysteine or cysteinylglycine (CysGly) provided by astrocytes (Sagara et al., 1993a; Dringen et al., 1999; Wang and Cynader, 2000; Dringen and Hirrlinger, 2003). Astrocytes export GSH into the extracellular space through multidrug resistance protein-1 (Mrp-1) (Minich et al., 2006), where it can be broken down by γ-glutamyl-transpeptidase into glutamate and CysGly

(Hanigan and Rickets, 1993). Aminopeptidase-N, expressed on the cell membrane of neurons, then breaks CysGly down into glycine and cysteine (Dringen et al., 2001). Neurons then take up the extracellular cysteine through EAAT3 to synthesize GSH (Sagara et al., 1993a; Zerangue and Kavanaugh, 1996b; McBean, 2002). Without the GSH/cysteine supplied by astrocytes, neurons are much more susceptible to oxidative insults (Drukarch et al., 1997; Abramov et al., 2003; Shih et al., 2003). GSH metabolism is illustrated in Figure 1.5.

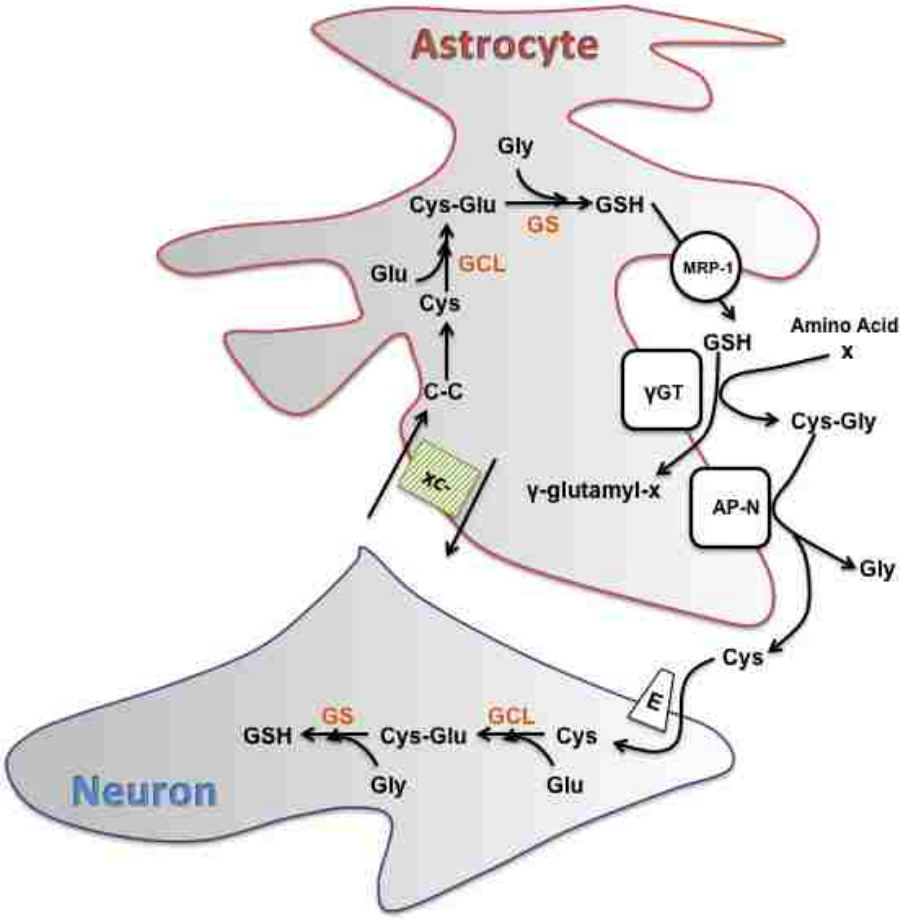

Figure 1.5 Interaction between astrocytes and neurons: GSH metabolism. Cystine (C-C) enters astrocytes through system x_c- and is rapidly reduced to cysteine (cys). Glutamate-cysteine ligase (GCL) catalyzes glutamate and cysteine to γ-glutamylcysteine (Cys-Glu). Glycine (Gly) is added to the dipeptide by GSH synthase (GS) to form the tripeptide glutathione (GSH). Astrocytes then export GSH through multidrug resistance protein 1 (MRP-1) into the extracellular space where it is broken down into cysteinylglycine (Cys-Gly) by γglutamyltransferase (γGT). It is further broken down into glycine and cysteine by aminopeptidase (AP-N). Cysteine can then be taken up into neurons via EAAT3 (E), where it is metabolized into GSH. Adapted and modified from (Bridges et al., 2012)

Although GSH is the main antioxidant in the brain, compared to other tissues the concentration of GSH is low. The concentration also varies between cells types; for example, the concentration in neurons is ~2.5mM and in astrocytes it is ~3.8mM (Rice

and Russo-Menna, 1998) . The brain's high oxygen consumption and low concentration of GSH makes it, particularly the neurons, susceptible to oxidative stress.

TRANSFORMING GROWTH FACTOR-β1

Transforming growth factor-β1 (TGF-β1), first discovered in the early 1980s, is a cytokine with wide ranging effects both in development and in the adult brain, including regulating cellular processes like growth and development (Böttner et al., 2000; Massagué et al., 2000), differentiation (Ishihara et al., 1994), and apoptosis (Prehn et al., 1994; Zhu et al., 2001; 2002). TGF-β1 is one of three TGF-β isoforms and is part the TGF-β superfamily, which includes multiple cytokines. Its expression is very low during development and in the adult brain (Flanders et al., 1991; Pelton et al., 1991; Unsicker et al., 1991). While some studies show it is mainly confined to the meninges and choroid plexus (Unsicker et al., 1991), others have found it is present in the cerebral cortex (Vivien et al., 1998).

Receptors and Intracellular Signaling

When TGF-β1 is first synthesized it becomes part of a large latent complex (LLC) contained in the cell's extracellular matrix (Akhurst and Hata, 2012). The complex is composed of a TGF-β1 homodimer, a latency-associated peptide (LAP), and a latent TGFβ binding protein (LTBP). When TGF-β1 is activated the LLC is released from the extracellular matrix and the LAP and LTBP are broken down, releasing TGF-β1. Upon its release TGF-β1 is able to interact with its receptors: TGF-β receptor I and II (TGF-βRI, -II) (Massagué, 1998). Both TGF-βRI and TGF-βRII are serine/threonine kinases and are expressed on neurons, astrocytes, and microglia throughout the brain (Böttner et

al., 1996; Vivien et al., 1998; Vivien and Ali, 2006). There are two TGF-βRI isoforms that TGF-β1 signals through: activin receptor-like kinase 1 (ALK1) and ALK5 (Massagué, 1998; Akhurst and Hata, 2012). Signaling begins when TGF-β1 binds to TGF-βRII, which recruits and phosphorylates TGF-βRI. Following activation, TGF-βRI phosphorylates and activates receptor specific signaling mother against decapentaplegic (R-SMAD) proteins, which then associate with its common mediator SMAD (C-SMAD). Together R-SMAD and C-SMAD translocate to the nucleus where they regulate gene transcription (Figure 1.6).

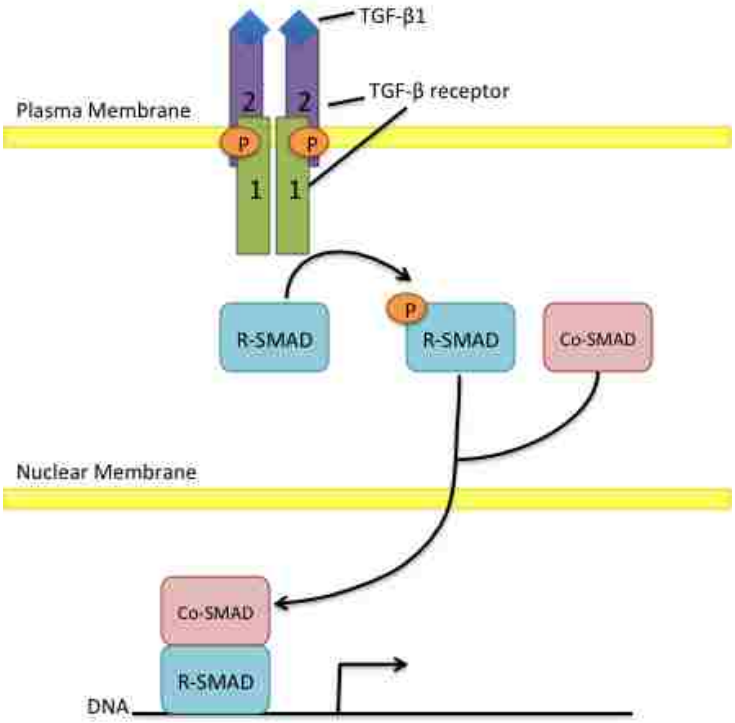

Figure 1.6 The canonical TGF-β1 signaling pathway. Adapted and modified from (Massagué, 2000)

Recently other signaling pathways of TGF-βRI/II activation have begun to emerge (Figure 1.7) (Massagué, 2000; Vivien and Ali, 2006; Akkhurt and Hata, 2012). Through crosstalk these non-canonical pathways and the SMAD pathways can modulate

each other. Also, along with being serine/threonine kinases, recently it was discovered that TGF-βRI/II can also phosphorylate tyrosine, which plays a role in activating the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) signaling pathway (Lee et al., 2007; Zhang, 2009).

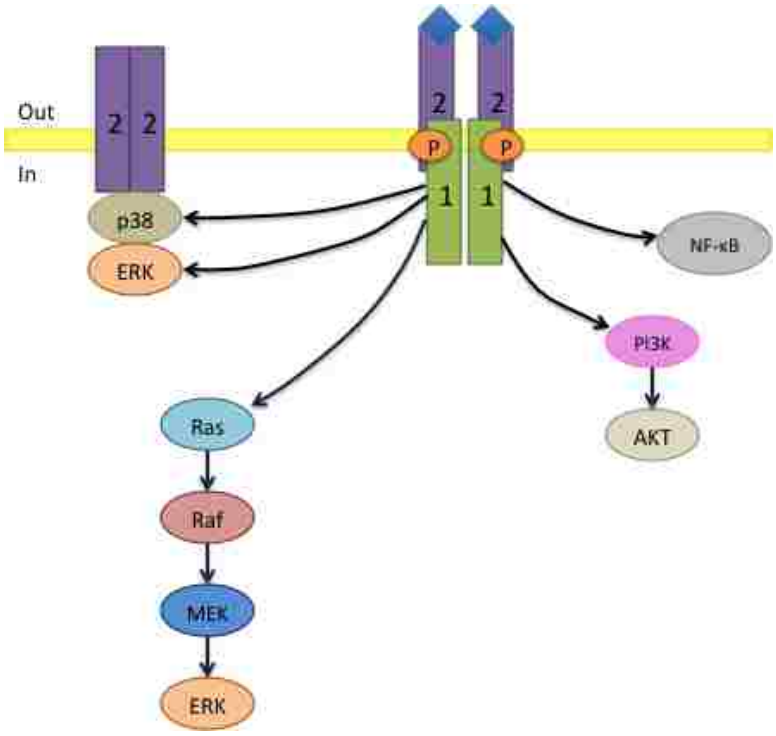

Figure 1.7 The non-canonical TGF-β1 signaling pathway. Adapted and modified from (Akhurt and Hata, 2012)

TGF-β1 in Brain Disorders

While TGF-β1 expression is very low in the adult brain (Flanders et al., 1991; Pelton et al., 1991; Unsicker et al., 1991) in response to injury and neurodegeneration TGF-β1 is highly upregulated and secreted by many different cell types (Pratt and McPherson, 1997; Flanders et al., 1998; Vivien and Ali, 2006), suggesting it plays a role in regulating neuronal survival and death. In fact TGF-β1 knockout mice have

widespread neuronal degeneration (Brionne et al., 2003), which reveals a role for TGF-β1 in regulating neuronal survival. Below is a brief description of some of the diseases TGF-β1 has been shown to be upregulated in and the role it plays in neuronal survival/death.

Amyotrophic Lateral Sclerosis

TGF-β1 is increased in both the SOD1-G93A mouse model of amyotrophic lateral sclerosis (ALS) and in the cerebral spinal fluid (CSF), plasma, and ventral horn of ALS patients; levels of TGF-β1 are positively correlated with the duration of the disease (Houi et al., 2002; Iłzecka et al., 2002; Endo et al., 2015). In SOD1-G93A mutant mice, overproduction of TGF-β1 in astrocytes resulted in a decreased inflammatory reaction by microglia, which accelerated disease progression in a non-cell autonomous manner (Endo et al., 2015). This suggests that TGF-β1 negatively regulates disease progression. In further support of this, inhibiting TGF-β1 signaling led to a decrease in disease progression and extended the survival time in SOD1-G93A mice (Endo et al., 2015).

Alzheimer's Disease

While increased levels of TGF-β1 have been observed in in the CSF, serum, and even in the amyloid-β (Aβ) plaques of patients with Alzheimer's disease (AD) (van der Wal et al., 1993; Chao et al., 1994; Zetterberg et al., 2004), the effects of this increase are unknown. In fact, there is conflicting data as to whether increased TGF-β1 plays a role in driving disease pathology or is neuroprotective. Overexpression of TGF-β1 in mice has been shown to lead to overexpression of amyloid precursor protein (APP), which drives Aβ production in astrocytes (Lesné et al., 2003) and increases Aβ deposition in cerebral blood vessels and meninges (Wyss-Coray et al., 1997). These studies suggest that TGF-

β1 may enhance AD pathology. However, TGF-β1 has also been shown to be protective against Aβ toxicity in hippocampal cultures (Ren and Flanders, 1996) and in Aβ-induced AD mouse models (Shen et al. 2014; Chen et al., 2015). Beneficial effects of TGF-β1 in these models included decreases in production of pro-inflammatory cytokines (Chen et al., 2015), cognitive deficits, glial activation, APP expression, and neuronal loss (Shen et al., 2014). Other protective effects of TGF-β1 on Aβ toxicity include preserving mitochondrial potential (Prehn et al., 1996), inhibiting apoptosis by regulating gene expression to favor the anti-apoptotic pathway (Kim et al., 1998), and even increasing the clearance of Aβ (Wyss-Coray et al., 2001).

Ischemia

TGF-β1 has been well studied in models of ischemia. It is upregulated in the infarct and the area surrounding the vessel occlusion (penumbra) in the brains of stroke patients (Krupinski et al., 1996). Similarly, following models of focal ischemia in rats TGF-β1 is also upregulated (Lehrmann et al., 1998; Ruocco et al., 1999) and exerts neuroprotective effects. For example, injection of TGF-β1 into the ventricles before the induction of ischemia decreased brain lesion size (Prehn et al., 1993; Henrich-Noack et al., 1996). TGF-β1 overexpression decreased chemokine expression and infarct size following middle cerebral artery occlusion (MCAO) and reperfusion (Pang et al., 2001), while blocking endogenously released TGF-β1 following MCAO increased infarct size (Ruocco et al., 1999).

Two of the key mechanisms involved in cell death following ischemia are excitotoxicity and apoptosis. The effects TGF-β1 exerts on insults that induce excitoxicity or apoptosis have been studied *in vitro*, in order to better understand the

mechanism behind its effects on neuronal death. TGF-β1 exerts opposite effects on slowly-triggered vs rapidly-triggered excitotoxicity. TGF-β1 is protective against short applications of high doses of glutamate or NMDA (rapidly-triggered excitotoxicity) in hippocampal and cortical neurons (Prehn and Krieglstein, 1994; Prehn and Miller, 1996; Bruno et al., 1998). However, TGF-β1 exacerbates the toxicity of long applications of low doses of glutamate (slowly-triggered excitotoxicity) (Prehn and Miller, 1996). TGF-β1's protection against NMDA receptor mediated toxicity involves upregulation of type 1 plasminogen activator inhibitor (PAI-1) (Buisson et al., 1998; Docagne et al., 1999). The protection by PAI-1 is due to its inhibition of tPA, a protein that increases NMDA toxicity by cleaving the NR1 subunit leading to increased NMDA induced calcium influx (Nicole et al., 2001). The mechanism behind exacerbation of slowly-triggered excitotoxicity is unknown.

TGF-β1 protects against apoptosis by regulating the expression of apoptotic genes. For example, TGF-β1 pretreatment increased expression of the anti-apoptotic gene, B-cell lymphoma 2 (Bcl2) and Bcl-xl, in hippocampal neurons deprived of trophic factors (Prehn et al., 1994). TGF-β1 is also able to inhibit the pro-apoptotic gene caspase-3 and protect hippocampal neurons from staurosporine-induced apoptosis (Zhu et al., 2001). *In vivo*, TGF-β1 is able to reduce ischemic lesions and neurological deficits by inhibiting the pro-apoptotic genes Bad and caspase-3 induced by MCAO; TGF-β1's effects are through activation of the MAPK/ERK pathway (Zhu et al., 2002).

SYSTEM X_C^-

The cystine/glutamate antiporter (system x_c^-) is a sodium-independent, chloride-dependent amino acid transporter system localized in the plasma membrane. First

characterized in human fibroblast cell cultures, system x_c^-, is an antiporter that mediates the uptake of cystine into cells in exchange for exporting glutamate from the cell in a 1:1 ratio (Bannai and Kitamura, 1980; Bannai, 1986).

Structure of System x_c^-

System x_c^- is a member of the solute carrier (SLC) 7 family of amino acid transporters and is a heterodimer composed of two subunits: a 502 amino acid short chain protein xCT and 4F2hc, the heavy chain subunit. 4F2hc is found in multiple members of the SLC7 family of transporters and is responsible for the transport of the antiporter to the plasma membrane (Estévez et al., 1998; Sato et al., 1999; Bassi et al., 2001; Verrey et al., 2004). The 4F2hc subunit is linked by a disulfide bridge to the xCT subunit (Torrents et al., 1998; Shih et al., 2006). xCT is encoded by the SLC7a11 gene (Bassi et al., 2001) and is the functional subunit that confers substrate specificity of the transporter (Sato et al., 1999; Bassi et a., 2001). It is composed of 12 transmembrane domains and both the N- and C-terminus are located in the cytoplasm (Gasol et al., 2004) (Figure 1.8).

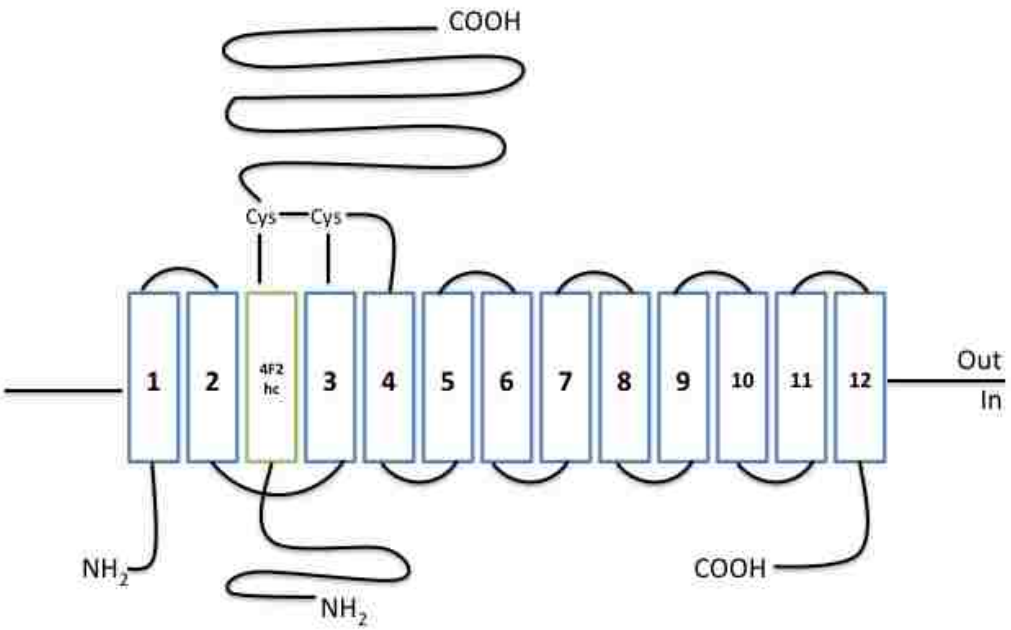

Figure 1.8 The structure of system x_c-. Adapted and modified from (Shih et al., 2006)

Function of System x_c-

System x_c- has three main functions. Due to its dual action of importing one molecule of cystine in exchange for exporting one molecule of glutamate, it is able to 1) regulate intracellular GSH levels and 2) regulate extracellular glutamate concentrations. Often overlooked because of its first two functions, system x_c- also 3) plays a role in regulating the redox environment of the cell. Each of these functions is described in more detail below.

Regulation of GSH Levels

As previously described, GSH is essential for the reduction of ROS in the brain and protection from oxidative stress; system x_c- not only provides astrocytes with the substrate to synthesize GSH, but it is also critical for GSH synthesis in neurons. Certain cells rely heavily on system x_c- to protect them from oxidative glutamate toxicity.

Oxidative glutamate toxicity occurs when high levels of extracellular glutamate are applied to cells that lack glutamate receptors; instead of causing excitotoxicity, high levels of glutamate competitively inhibit the uptake of cystine through system x_c^-, causing GSH depletion, resulting in oxidative stress and cell death (Miyamoto et al. 1989; Murphy et al. 1989; 1990; Sagara et al. 1993b; Ratan et al. 1994). Overexpression of xCT on astrocytes can protect immature cortical neurons from oxidative glutamate toxicity (Shih et al., 2006).

System x_c^- activity is also needed in order to maintain the high levels of GSH necessary for glioma cells to grow (Chung et al. 2005; Chung and Sontheimer 2009); that is why when cellular GSH levels are depleted by diethyl maleate (DEM) treatment, glioma cells increase xCT expression and system x_c^- activity (Kim et al. 2001). Upregulation of system x_c^- can also protect cells from different types of oxidative stress. For example, insulin-like growth factor 1 (IGF-1) and TGF-β upregulate system x_c^- activity and protect dental pulp cells against oxidative stress induced by dental materials (Pauly et al., 2011). Interleukin-1β (IL-1β) upregulates system x_c^- activity, which protects astrocytes from ROS-inducing $FeSO_4$ and tert-butyl hydroperoxide (tBOOH) exposure (He et al., 2015). On the other hand, astrocytes derived from mice with the subtle gray pigmentation mutant phenotype (sut/sut), an xCT loss of function mutation, have reduced proliferation as a result of decreased GSH and increased oxidative stress (Shih et al., 2006). Together these studies show that upregulating system x_c^- confers protection against oxidative stress in the cells in which it is upregulated by increasing cystine uptake, which leads to the production of the antioxidant GSH.

Extracellular Glutamate Regulation

The second main function of system x_c^- is the regulation of extracellular glutamate concentrations. Non-vesicular release of glutamate through system x_c^- is the major source of extracellular glutamate in several rodent brain regions (De Bundel et al., 2011; Massie et al., 2011; Baker et al. 2002) and it is able to regulate neuronal activity through extrasynaptic glutamate receptors. For example, glutamate released by system x_c^- can activate presynaptic mGluR2/3 receptors and inhibit synaptic glutamate release (Baker et al., 2002; Moran et al., 2005). It can also change receptor levels in the synapse, which can affect synaptic strength (Augustin et al., 2007; Williams and Featherstone, 2014). Sut/sut mice have impaired long-term potentiation (LTP) and deficits in their long-term memory (Li et al., 2012) and $xCT^{-/-}$ mice have decreased extracellular glutamate and impaired spatial working memory (De Bundel et al., 2011).

In contrast to the possible neural protective mechanism of system x_c^- against oxidative stress, it has been shown that glutamate release via system x_c^- can cause excitotoxicity and lead to neuronal death. The strongest evidence comes from studies involving glial cells. Activation of microglia can increase glutamate release through system x_c^- causing toxicity to cerebellar granule cells (Piani and Fontana, 1994), cortical neurons (Qin et al., 2006), and oligodendrocytes (Domercq et al. 2007). System x_c^- on astrocytes has also been shown to play a role in neuronal death. Activation of astrocytes with IL-1β leads to increased system x_c^- mediated glutamate release causing enhanced excitotoxicity of cortical neurons exposed to hypoxia or glucose deprivation (Fogal et al., 2007; Jackman et al., 2010). And long-term exposure to fibroblast growth factor-2 (FGF-2) upregulates system x_c^- activity and causes AMPA receptor mediated excitotoxicity in

cortical neurons (Liu et al., 2012; 2014). Together these studies show that system x_c^- can increase extracellular glutamate levels, which can lead to excitotoxicity.

Regulation of the Redox Environment

Finally, system x_c^- is involved in regulating the redox environment around the cell membrane by maintaining proper levels of GSH and cystine/cysteine (Conrad and Sato, 2012; Lewerenz et al., 2013). GSH is exported from the cell by MRP-1 and contributes to the reduced extracellular environment (Wang and Cynader 2000; Dringen and Hirrlinger 2003). Likewise cysteine can also be directly exported from the cell via system ASC and oxidized to cystine. System x_c^- drives this cystine/cysteine cycle, which helps maintain the redox environment independent of the GSH (Bannai and Ishii 1982; Anderson et al. 2007). The redox state of the cell is important because it can play a large role on cell signaling. For example, the NMDA receptor has an extracellular redox site and changes in the redox environment can affect the open-channel frequency and potentiate or decrease the magnitude of the response (Aizenman et al., 1989; Tang and Aizenman, 1993). The redox state can also impact enzyme and transcription factor activity (Janssen-Heininger et al., 2008).

xCT knockout mice ($xCT^{-/-}$) provide evidence for the involvement of system x_c^- in redox balance. Sato et al. (2005) found that the plasma in $xCT^{-/-}$ mice is more oxidized, as a result of higher levels of cystine and lower levels of GSH. Also, fibroblasts cultured from these mice died unless the reducing agent 2-mercaptoethanol was added. If 2-mercaptoethanol was removed cysteine and GSH levels decreased and cells began to die within 24 hours.

Regulation of System x_c^-

System x_c^- has similar affinities for transport of both cystine and glutamate; therefore, the concentration gradient of these substrates determines the direction of transport (Lewerenz et al., 2013). Physiologically, cystine is found at very low concentrations inside the cell, while glutamate has high intracellular and low extracellular concentrations thus, intracellular concentrations of glutamate drive the release of glutamate from the cell through system x_c^-, which leads to the import of cystine (Lewerenz et al., 2013). EAATs regulate intracellular/extracellular glutamate concentrations and, therefore, regulate system x_c^- activity (Rimaniol et al., 2001; Lewerenz et al., 2006). Since system x_c^- can transport glutamate both directions, increases in extracellular glutamate can act as a competitive inhibitor of cystine uptake (Bannai, 1986).

Regulation of xCT subunit expression is more important in determining system x_c^- activity than the 4F2hc subunit (Lewerenz et al., 2013). The factors that regulate xCT and mechanisms behind its regulation are still largely unknown. Given its role in oxidative stress and excitotoxicity, along with its emerging role in many neurodegenerative diseases, understanding what regulates system x_c^- is important and may one day help in developing therapeutic agents for some of these diseases. Below is a brief description of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant responsive element (ARE) and the eukaryotic initiation factor-2 (eIF2α)-activating transcription factor (ATF) 4- amino acid response element (AARE) pathways, which regulate system x_c^- expression (Figure 1.9).

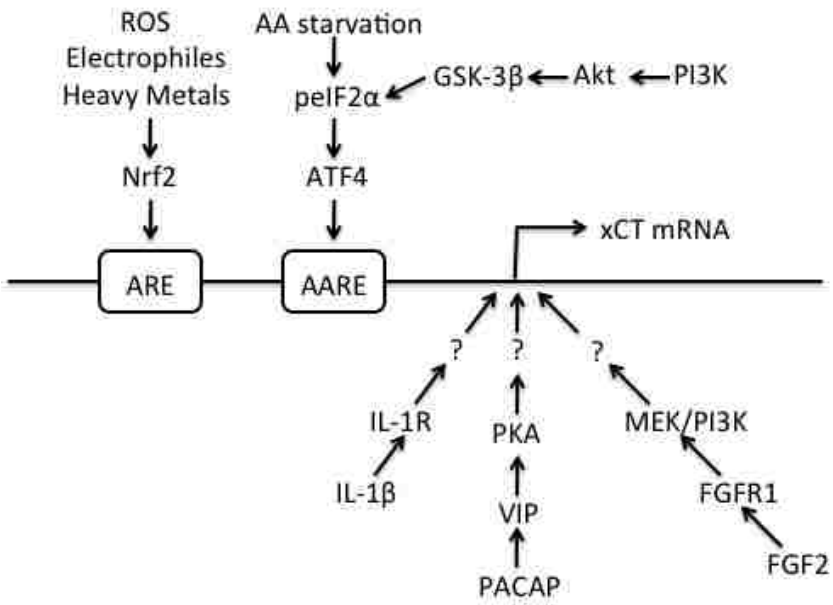

Figure 1.9 Factors and pathways that regulate the transcription of xCT. Adapted and modified from (Lewerenz et al., 2012)

Nrf2-ARE Pathway

Oxidative stress is one of the main regulators of system x_c-. Xanthine/xanthine oxidase induced oxidative stress in Müller glial cells causes an upregulation of system x_c- activity (Mysona et al., 2009). Also, oxidative stress and nitric oxide upregulate system x_c- activity in a retinal ganglion cell line (RGC-5) (Dun et al., 2006), while oxidative stress induced by exposure to DEM increases system x_c- function in human glioma cells (Kim et al., 2001).

In response to oxidative and electrophilic stress cells activate the Nrf2-ARE pathway, which increases the cell's antioxidant defenses (Itoh et al., 1997), including intracellular and extracellular GSH levels (Sasaki et al., 2002; Shih et al, 2006). Under normal conditions, Nrf2 is bound to kelch-like ECH-associated protein 1 (Keap1), which retains Nrf2 in the cytoplasm. However, in response to ROS and electrophiles Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to ARE sequences,

also known as electrophile response elements (EpRE), and activates gene transcription (Itoh et al., 2004; Taguchi et al., 2011; Kansanen et al., 2013). The Nrf2-ARE pathway is responsible for activating multiple genes involved in neuronal survival. Many of the genes activated are involved in the metabolism of GSH, including glutamate-cysteine ligase, glutathione synthetase, glutathione S-transferase, glutathione reductase, and multidrug resistance protein 1 (Sasaki et al., 2002; Lee and Johnson, 2004; Shih et al., 2006). Transcription of xCT, which contains four ARE sequences in the 5' end of its gene, is also activated (Sasaki et al., 2002).

Studies show that the Nrf2-ARE pathway can upregulate system x_c^- in response to a wide variety of stress agents, including diethyl maleate, H_2O_2, cadmium, and oxidative glutamate toxicity in different types of cells (Ishii et al., 2000; Sasaki et al., 2002; Lewerenz et al., 2009). It is important to note that the Nrf-2-ARE pathway is preferentially activated in astrocytes (Shih et al., 2006; Johnson et al., 2008) and upregulation of system x_c^- through this pathway not only protects astrocytes from oxidative stress, but also protects neighboring neurons (Shih et al. 2003).

eIF2α-ATF4-AARE Pathway

Amino acid deprivation causes an increase in system x_c^- activity through activation of the ATF4-AARE pathway (Sato et al., 2004). Low levels of amino acids activate the general control non-derepressible-2 (GCN2) protein kinase, which phosphorylates eIF2α (Zhang et al., 2002; Wek et al., 2006). Composed of eIF2α, eIF2β, and eIF2γ, eIF2 is part of the initiating complex for most protein synthesis. Phosphorylation of eIF2α leads to global inhibition of protein synthesis in order to slow down cellular activity and protect the cell (Wek et al., 2006; Kilberg et al., 2009). While

translation of most proteins is inhibited, eIF2α phosphorylation induces translation of select transcripts, like ATF4 (Vattem and Wek, 2004; Wek et al., 2006; Kilberg et al., 2009). ATF4 binds to the AARE sequence and results in translation of the gene (Kilberg et al., 2009). The 5' end of xCT contains two AARE sequences, thus ATF4 binding results in increased xCT transcription (Sato et al., 2004).

The ATF4-AARE pathway can also be activated by phosphatidylinositide 3-kinase (PI3K) in glioblastomas (Lewerenz et al., 2014). High frequency neuronal activation *in vitro* activates PI3K, which generates 3,4,5-triphosphate (PIP3) through phosphorylation of inositol lipids in the membrane (Lewerenz et al., 2014). PIP3 activation leads to Akt activation, which phosphorylates and inhibits glycogen synthase kinase 3β (GSK-3β) (Hers et al., 2011; Matsuda et al., 2013; Lewerenz et al., 2014). In turn, inhibition of GSK-3β leads to GCN2 activation and the eIF2α-ATF4-AARE pathway continues as previously described (Lewerenz et al., 2014).

In embryonic fibroblasts, mutation of the eIF2α phosphorylation site results in decreased ATF4 expression and system x_c^- activity (Lewerenz and Maher, 2009). Likewise, increasing eIF2α phosphorylation in hippocampal HT22 cells increases ATF4 expression and system x_c^- activity. The increase in system x_c^- activity led to increased GSH levels and protected against oxidative glutamate toxicity (Lewerenz and Maher, 2009). Together these studies show that basal levels of eIF2α phosphorylation help determine basal system x_c^- activity (Lewerenz and Maher, 2009).

Growth Factors and Other Peptides

Growth factors and other peptides also regulate system x_c^- activity. Our lab has previously shown that FGF-2 upregulates system x_c^- activity in astrocytes; upregulation

was through FGF receptor 1 (FGFR1) and both the MEK/ERK and PI3-kinase pathways (Liu et al., 2012). We have also shown that IGF-1 and TGF-β upregulate system x_c^- activity in dental pulp cells (Pauly et al., 2011).

Interleukin-1β (IL-1β) upregulates system x_c^- activity, specifically in cortical astrocytes, through the IL-1 receptor (Jackman et al., 2010). The same lab also showed that IL-1β upregulates GSH synthesis in astrocytes through nuclear factor-kappaB (NF-κB) activity (He et al., 2015). While the 5' end of the xCT gene contains a NF- κB binding site (Sato et al., 2001), there is no direct evidence that IL-1β increases system x_c^- activity through the NF- κB pathway.

Similar to FGF-2 and IL-1β, pituitary adenylate cyclase-activating polypeptide (PACAP) increases system x_c^- activity in cortical cultures. The pathway has been partially determined. Applying PACAP for at least 6 hours induces system x_c^- activity through VPAC1R, the vasoactive intestinal peptide (VIP) receptor, and a protein kinase A (PKA)-dependent pathway (Resch et al., 2014). It has also been shown that inhibiting PKA decreases system x_c^- activity in *ex vivo* striatal punches rapidly (Baker et al. 2002). The differences in the time course of the two studies suggest that PKA may regulate system x_c^- through multiple mechanisms (Massie et al., 2015). More direct evidence is needed to understand the mechanism(s) behind PKA's regulation of system x_c^- expression and activity.

Intracellular GSH levels can also increase system x_c^- activity. Depleting GSH from astrocytes that have been treated with dibutyryl-cyclic AMP (dbcAMP) upregulates system x_c^- activity. The exact mechanism is unknown, but it is independent of the Nrf2 pathway (Seib et al., 2011).

Potential Role of System x_c^- in Brain Disorders

Oxidative stress and excitotoxicity are two of the key mechanisms in many neurodegenerative diseases (Coyle and Puttfarcken, 1993; Simonian and Coyle, 1996; Doble, 1999). The dual nature of system x_c^- suggests that it may provide antioxidant protection during times of increased oxidative stress; however, by releasing glutamate into the extrasynaptic space it also has the potential to contribute to neuronal death through excitotoxicity. In this thesis we analyze the function of system x_c^- in the SOD1-G93A mutant mouse model of ALS. However, the pathology of many neurodegenerative diseases suggests that system x_c^- may also play an important role in them, so we briefly review what is known about system x_c^- in these diseases as well.

Amyotrophic lateral sclerosis

ALS is the most common paralytic disease in adults, characterized by the loss of motor neurons in the motor cortex, brainstem, and spinal cord, which results in loss of motor function. It is believed that approximately 10% of ALS cases are inherited, while the majority of cases are sporadic, and are of unknown cause. The mutation involved in approximately 10-20% of genetic cases involves copper/zinc superoxide dismutase type 1 (SOD-1). Interestingly, silencing the SOD-1 gene does not produce ALS symptoms (Shefner et al., 1999), but rather it appears to be a gain-of-function mutation as mice expressing the human SOD-1 mutation do develop ALS-like symptoms (Gurney et al. 1994). Also, motor neuron death occurs through a non-cell autonomous mechanism with glial cells (Clement et al., 2003; Boillée et al., 2006; Yamanaka et al., 2008). Although it is unclear what causes ALS, it is clear that oxidative stress is a key mechanism involved

in the pathology of the disease. Elevated levels of protein carbonyl groups, markers of oxidative damage, have been found in post mortem tissue from ALS patients (Bowling et al., 1993; Ferrante et al., 1997); and increased production of both oxygen and hydroxyl free radicals have been found in transgenic mice with the human SOD1-G93A mutation (Yim et al., 1996; Bogdanov et al., 1998; Liu et al., 1998).

Another key mechanism in the pathology of ALS is excitotoxicity. Elevated levels of glutamate have been found in patients with ALS (Spreux-Varoquaux et al., 2002). Previous research has shown that the increase in glutamate levels may be attributed to a decrease in EAAT2/GLT-1, the main mechanism by which glutamate is taken up out of the extrasynaptic space and into astrocytes. Decreased levels of GLT-1 have been reported in both the brain and spinal cord in post mortem tissue of ALS patients (Rothstein et al., 1995) and in the ventral horn of SOD1-G93A transgenic rat and mouse spinal cords (Canton et al., 1998; Warita et al., 2002; Bendotti et al., 2001). While impaired glutamate transports seem to play an important role in the increased extracellular glutamate levels seen in ALS, decreases in the glutamate transporter do not appear until after the onset of symptoms in the mouse models and, therefore, are probably not the primary cause of motor neuron loss (Canton et al., 1998; Warita et al., 2002; Bendotti et al., 2001).

Elevated levels of glutamate could also occur as a result of increased system x_c^- function and/or expression. Prior to our work done in *ex vivo* slice from SOD1-G93A mice (Chapter II), the function of system x_c^- had not been assessed in ALS. Following our study, system x_c^- function was also examined in the SOD1-G37R mouse model, a much slower progression model of the disease. In the SOD1-G37R model xCT mRNA

increased in the spinal cord over the course of the disease, specifically in microglia (Mesci et al., 2015). Post-mortem spinal cord tissue from ALS patients also expressed xCT mRNA that correlated with a marker for macrophage inflammation (Mesci et al., 2015). Interestingly, deletion of xCT in the SOD1-G37R mouse led to an earlier onset of symptoms followed by a prolonged symptomatic stage; at end stage of the disease there were more surviving motor neurons (Mesci et al., 2015). The implications of the findings in this study will be discussed further in the context of our discoveries in the SOD1-G93A model (Chapter V).

Alzheimer's disease

AD is the most common neurodegenerative disease. It is characterized by degeneration in the hippocampus and cerebral cortex, which leads to impairments in memory and cognition. Two of the main hallmarks of AD are the formation of extracellular Aβ plaques and intracellular tangles of phosphorylated tau protein. Aβ is derived from cleavage of the amyloid precursor protein (APP) (Selkoe, 1994) and abnormal accumulation can lead to cell death by inducing oxidative stress (Varadarajan et al., 2000; Butterfield et al. 2002; Butterfield and Lauderback, 2002).

Reactive microglia are found in the plaques of AD patients. Therefore, the first studies that analyzed system x_c^- function in AD looked at the effects of soluble APP (sAPP) and $Aß_{1-40}$ on primary microglia cultures. Both sAPP and $Aß_{1-40}$ peptide increased glutamate release from microglia through system x_c^- (Barger and Basile, 2001; Qin et al., 2006). The sAPP induced increase in glutamate release caused increased calcium elevation in hippocampal neurons and compromised their synaptic density (Barger and Baile, 2001), while $Aß_{1-40}$ induced glutamate release caused NMDA receptor

mediated excitotoxicity and masked the protective effects of microglia release of apolipoprotein E (apoE) (Qin et al., 2006). These studies suggest that increased system x_c^- activity in microglia has detrimental effects on neurons in AD. Another study, however, found that increasing system x_c^- by activating the eIF2α-ATF4-AARE pathway made PC12 cells more resistant to $Aß_{1-42}$ peptide toxicity (Lewerenz and Maher, 2009).

To date there are only a couple of *in vivo* studies that have assessed system x_c^- in AD. In 18-month old AßPP23 mice expression of xCT, as assessed by western blot, is increased in the cortex. At this same time point, GLT-1 expression was decreased and there were increased levels of extracellular glutamate (Schallier et al., 2011). Transgenic mice expressing human APP and wild-type mice injected with $Aβ_{1-40}$ have increased xCT mRNA levels in microglia in amyloid plaques (Qin et al., 2006).

There have been some AD studies assessing the effects of N-acetylcysteine (NAC). NAC is a pro-cysteine drug that drives system x_c^- activity (Kupchik et al. 2012). In one double-blind study where AD patients were given NAC, they showed beneficial trends in all areas tested, particularly in cognitive tasks (Adair et al., 2001). NAC pretreatment was also shown to protect SHS5Y neuroblastoma cells from $Aβ_{1-42}$ and $Aβ_{25-35}$ (Olivieri et al., 2001), an effect associated with decreased Aβ secretion and tau phosphorylation; they did not assess system x_c^- function in these cells. Therefore, while these studies show beneficial effects of NAC in AD none of those benefits have been directly linked to increased system x_c^- activity. Further studies using xCT knockout mice in AD mouse models may help to better clarify the role of system x_c^- in AD.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease characterized by a loss of dopaminergic neurons in the substantia nigra. Loss of dopaminergic neurons leads to a progressive loss of motor function; symptoms include stiff gait, tremors, impaired balance, slow movement, and eventually muscle atrophy (Beitz, 2014). While the cause of dopaminergic cell death is unknown, oxidative stress (Hwang, 2013) and excitotoxicity (Caudle and Zhang, 2009) play a role in the pathology of PD.

Determining the effects of system x_c^- in PD models has led to mixed results. In the hemi-Parkinson rat model, injection of 6-hydroxydopamine (6-OHDA) caused an ipsilateral increase in xCT protein in the striatum three weeks after injection; twelve weeks after injection xCT levels were back down to normal (Massie et al., 2008). In a follow up study dopaminergic neurons in the substantia nigra pars compacta were protected from 6-OHDA injection in $xCT^{-/-}$ mice compared to wild-type mice (Massie et al., 2011). GSH levels were unaffected and oxidative stress was not elevated in the $xCT^{-/-}$ mice; however, extracellular glutamate levels were significantly reduced (Massie et al., 2011). Taken together the data suggests that system x_c^- may contribute to the neurodegeneration seen in PD. A more recent study has yielded different results in the 6-OHDA model of PD. The anti-epileptic drug levetiracetam (LEV) increased xCT expression and GSH levels in astrocytes cultured from the striatum. Culture medium taken from LEV treated astrocytes was able to protect dopaminergic neurons from 6-OHDA toxicity, an affect that was blocked by xCT inhibitor SSZ (Miyazaki et al., 2016). *In vivo* LEV also increased xCT expression in striatal astrocytes and significantly decreased dopaminergic cell loss in mice injected with 6-OHDA (Miyazaki et al., 2016).

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD has also yielded different results regarding the actions of system x_c-. xCT expression was increased in the striatum, but decreased in the substantia nigra pars compacta following MPTP injection (Bentea et al., 2015). Also, MPTP injection resulted in similar levels of dopaminergic cell loss and similar behavioral deficits in both $xCT^{-/-}$ mice and wild-type mice (Bentea et al., 2015), which suggests system x_c- does not affect neurodegeneration of dopamine neurons. Given the differences seen between different models of PD and even within the same model, further studies need to be performed in order to gain a better understanding of system x_c-'s role in PD.

Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune demyelinating disease. Pathology of the disease includes white matter scar formation, inflammation, disruption in the blood brain barrier, and glutamate dysregulation (Werner et al., 2001; Minagar and Alexander, 2003; Goldenberg, 2012). Dysregulation of glutamate contributes to the death of oligodendrocytes, myelin-producing cells in the CNS, however the source of this glutamate is unknown (Werner et al., 2001).

In microglia-oligodendrocyte cocultures, activation of microglia by lipopolysaccharide (LPS) caused an increase in glutamate release through system x_c-, resulting in increased extracellular glutamate and oligodendrocyte toxicity (Domercq et al., 2007). Similarly, CD4+ T helper cells provoke microglia to release glutamate through system x_c- resulting in oligodendrocyte excitotoxicity (Evonuk et al., 2015).

Microglia cells from the spinal cord of rats treated with experimental autoimmune encephalomyelitis (EAE), a model of MS, have increased xCT mRNA and protein levels

(Pampliega et al., 2011). This finding correlates with increased xCT mRNA levels found in the leukocytes and optic nerve tissue from human MS patients (Pampliega et al., 2011). Blocking system x_c^- in both the chronic and relapse-remitting EAE models of MS decreased the clinical severity of the disease and decreased spinal cord demyelination (Evonuk et al., 2015). Likewise, mice that contain inactive system x_c^- due to a mutation in the xCT gene (sut/sut mice) are resistant to EAE treatment (Evonuk et al., 2015). Together this data suggests that increased glutamate release though system x_c^- may contribute to the degeneration of oligodendrocytes and that decreasing system x_c^- activity may be a therapeutic target for the treatment of MS.

Huntington's Disease

Huntington's disease (HD) is a fatal neurodegenerative disease caused by a polyglutamine expansion in the huntingtin (htt) gene. This autosomal dominant mutation leads to the death of GABAergic medium spiny neurons, mainly in the striatum; the mechanism of this selective neuronal death is unknown (Vonsattel and DiFiglia, 1998). However, there is strong evidence that there is mitochondrial dysfunction, resulting in defects in energy metabolism, oxidative stress, and excitotoxicity (Browne and Beal, 2004).

Little is known about system x_c^- activity in HD. In the striatal cell line $STHdh^{Q111/Q111}$, a model of HD, system x_c^- mRNA levels and protein expression were decreased, which resulted in decreased system x_c^- function (Frederick et al., 2014). These cells also have decreased GSH levels, increased levels of ROS, and are more sensitive to oxidative stress caused by DEM (Frederick et al., 2014). System x_c^- mRNA and protein levels were also decreased in the striatum of R6/2 mutant htt exon 1

transgenic mice, another model of HD (Frederick et al., 2014). Administration of NAC in the R6/1 model of HD resulted in delayed symptom onset and progression of motor deficits (Wright et al., 2015). The mechanism behind the benefits of NAC was not determined. Ceftriaxone, an antibiotic that increases xCT and EAAT2/GLT-1 expression (Lewerenz et al., 2009), also attenuated the R6/2 behavioral phenotype (Miller et al., 2008). The authors attributed ceftriaxone's positive effects to restoring basal levels of glutamate in the striatum through increased in GLT-1 expression, however, they did not assess xCT levels, so an increase in system x_c- activity contributing to the effects cannot be ruled out.

CHAPTER II

REGULATION OF SYSTEM X_C- IN THE SOD1-G93A MOUSE MODEL OF ALS

Abstract

The cystine/glutamate antiporter (system x_c-) is critical for the generation of the antioxidant glutathione by transporting cystine into the cell. At the same time, system x_c- also releases glutamate, which can potentially lead to excitotoxicity. The dual actions of system x_c- make it of great interest in any disease, like amyotrophic lateral sclerosis (ALS), in which there is evidence of the involvement of both oxidative stress and excitotoxicity. The present study investigated the regulation of system x_c- in the spinal cord of the SOD1-G93A transgenic mouse model of ALS. In spinal cord slices of 70 day old SOD1-G93A transgenic mice cystine uptake by system x_c- was significantly increased compared to age matched nontransgenic mice; but it was not significantly different at 55, 100, or 130 days. The spinal cord slices of 70 day old SOD1-G93A transgenic mice also showed significantly increased glutamate release in the presence of cystine. In contrast, in glial cells cultured from postnatal day 1-3 mice there was no difference in cystine uptake in cultures from SOD1-G93A mice; and it was actually less when oxidative stress was induced by exposure to iron. D-aspartate uptake through excitatory amino acid transporters (EAATs), the main mechanism by which glutamate is cleared from the extracellular space was also examined. In spinal cord slices of 70 day old SOD1-G93A mice no change in D-aspartate uptake was found. Together, these findings suggest that at 70 days of age, SOD1-G93A transgenic mice have increased system x_c- activity, but no change in EAAT function. These results raise the possibility

that excitotoxicity in the SOD1-G93A transgenic mouse, at least at early time points, may be due to increased system x_c^- activity and not decreased EAAT function.

Introduction

Amyotrophic lateral sclerosis is the most common paralytic disease in adults, characterized by the loss of motor neurons in the motor cortex, brainstem, and spinal cord, which results in atrophy and loss of motor function. It is believed that approximately 10% of ALS cases are inherited, while the majority of cases are sporadic. The mutation involved in approximately 10-20% of genetic cases involves copper/zinc superoxide dismutase type 1 (SOD-1). Interestingly, silencing the SOD-1 gene does not produce ALS symptoms (Shefner et al., 1999), but mice expressing the human SOD-1 mutation do develop ALS like symptoms (Gurney et al., 1994). Although it is unclear what causes ALS, it appears that oxidative stress plays a key role in the pathology of the disease. Elevated levels of protein carbonyl groups, markers of oxidative damage, have been found in post mortem tissue from ALS patients (Bowling et al., 1993; Ferrante et al., 1997); and increased production of oxygen free radicals have been found in transgenic mice with the human SOD1-G93A mutation (Yim et al., 1996; Bogdanov et al., 1998; Liu et al., 1998).

Another key mechanism in the pathology of ALS is excitotoxicity. Elevated levels of glutamate have been found in patients with ALS (Rothstein et al., 1990; Spreux-Varoquaux et al., 2002). Previous research has shown that the increase in glutamate levels may be attributed to a decrease in the high affinity glutamate transporter (EAAT2/GLT-1), the main mechanism by which glutamate is taken up from the extrasynaptic space and into astrocytes. Decreased levels of EAAT2/GLT-1 have been

reported in both the brain and spinal cord of post mortem tissue of ALS patients (Rothstein et al., 1995) and in the spinal cord of SOD1-G93A transgenic rats (Howland et al., 2002) and mice (Bendotti et al., 2001).

The cystine/glutamate antiporter (system x_c-) is a sodium-independent amino acid transporter that may play a key role in both oxidative stress and excitotoxicity due to its dual function. System x_c- mediates the transport of cystine into the cell in exchange for releasing glutamate into the extrasynaptic space. After being taken up into the cell, cystine is reduced to cysteine and used in the synthesis of glutathione (GSH), an abundant and important antioxidant in the brain (Dringen and Hirrlinger, 2003). This activity occurs mainly on non-neuronal cells and the release of GSH by these cells can be protective to neurons (Shih et al., 2003).

Not only does system x_c- act to prevent oxidative stress, but such stress appears to be the main trigger for its upregulation. Oxidative stress induced by exposure of Muller glial cells to xanthine/xanthine oxidase causes an upregulation of system x_c- activity (Mysona et al., 2009). Also, oxidative stress and nitric oxide upregulates system x_c- activity in a retinal ganglion cell line (RGC-5) (Dun et al., 2006), while oxidative stress induced by exposure to diethylmaleate increases system x_c- function in human glioma cells (Kim et al., 2001). Thus, it appears that upregulation of system x_c- may act as a mechanism of cellular protection against oxidative stress.

In contrast to the possible neural protective mechanism of system x_c- against oxidative stress, it is known that glutamate release via system x_c- can cause excitotoxicity and lead to neuronal death. The strongest evidence comes from studies involving glia. Glutamate release via system x_c- from microglia kills cerebellar granule cells (Piani and

Fontana, 1994). Also, activation of microglia by amyloid-β causes increased glutamate release and neuronal death in cortical cultures (Qin et al., 2006). System x_c^- on astrocytes may also play a role in neuronal death. Activation of astrocytes with IL-1β leads to increased system x_c^- mediated glutamate release causing enhanced excitotoxicity of cortical neurons exposed to hypoxia or glucose deprivation (Fogal et al., 2007; Jackman et al., 2010).

Due to the complex nature of the pathology of ALS, and the ability of system x_c^- to alter both oxidative stress and excitotoxicity, the current studies were designed to evaluate the function of system x_c^- in spinal cord slices of the SOD1-G93A transgenic mouse. Since oxidative stress is a main factor that can lead to the upregulation of system x_c^- it was our prediction that system x_c^- would be upregulated in these mice. We also evaluated the function of EAATs since no functional analysis of EAATs has been previously performed in spinal cord slices of SOD1-G93A transgenic mice. Finally, we cultured spinal cord glial cells to further characterize the regulation of system x_c^- in SOD1-G93A mice.

Materials and Methods

Materials

Male B6SJL-TgN(SOD1-G93A)1Gur/J and female B6SJLF1/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). ^{14}C-cystine was from PerkinElmer (Waltham, MA). All other chemicals were from Sigma (St. Louis, MO).

Animals

Male B6SJL-TgN(SOD1-G93A)1Gur/J autosomal hemizygous mice were bred with female B6SJLF1/J hybrid non-affected control mice. At 3 weeks of age, pups were

separated and housed individually. Mice were handled in accordance with a protocol approved by our institutional animal care committee.

Genotyping

The presence of the human G93A transgene was confirmed using PCR of DNA extracted from ear punch samples or tail snips. Ear punches and tail snips were dissolved in 50mM Tris, 2mM NaCl, 10mM EDTA, 1% sodium dodecyl sulfate (SDS), and 1mg/mL Proteinase K in a 56ºC water bath. They were then heat shocked for 8 minutes at 100ºC and immediately put on ice. 250µL of water was added and samples were stored overnight at 4ºC. PCR was then performed to identify transgenic littermates (Transgene forward primer: CAT CAG CCC TAA TCC ATC TGA; Transgene reverse primer: CGC GAC TAA CAA TC A AAG TGA).

Neurological Scoring

Neurological scoring was performed three times a week, starting around day 55 and was performed as recommended by the Jackson Labs guide "Working with ALS mice". The score criteria was as follows:

Score of 0: Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds, suspended two to three times.

Score of 1: Collapse or partial collapse of leg extension towards lateral midline or trembling of hind legs during tail suspension.

Score of 2: Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table.

Score of 3: Rigid paralysis or minimal joint movement, foot not being used for generating forward motion.

Score of 4: Mouse cannot right itself within 30 seconds after being placed on either side.

A score of 0 is pre-symptomatic. A score of 1 is early symptomatic. A score of 2 or 3 is symptomatic and a score of 4 results in termination.

Spinal Cord Removal and Slice Preparation

Experiments were performed using spinal cord slices from transgenic and littermate nontransgenic mice. Male and female mice of various ages were euthanized by cervical dislocation following isoflurane anesthesia. The entire vertebral column from just below the skull to the hips was removed and placed into ice cold slice buffer (119 mM NaCl, 3mM KCl, 1.4mM KH_2PO_4, 2.7mM $MgSO_4$, 26mM $NaHCO_3$, 7.8mM glucose, 2.4mM $CaCl_2$). A 16-gauge beveled needle was inserted into the lumbar end of the column and the spinal cord was ejected from the vertebral column by injecting slice buffer into the vertebral column (Meikel and Martin, 1981). Using a McIlwain tissue chopper, the spinal cord was cut transversely into 400μm slices and placed onto nylon mesh platforms in ice cold slice buffer. Slices from the cervical, thoracic, and lumbar levels of the spinal cord were used for experiments. Slices were then incubated in slice buffer with 95% O_2 and 5% CO_2 at 37°C for 30 minutes before uptake or release experiments. Alternating slices were used for the different experimental conditions, with 2-3 slices for each condition being used to generate each "n" value; multiple animals were used.

Spinal Cord Cell Culture

Astrocyte-enriched glial cultures from the spinal cord were prepared from postnatal day 1-3 SOD1-G93A mice as previously described, with modifications (Kerstetter and Miller, 2012). Briefly, dissociated spinal cord cells were plated on 24-well plates (2.0 cm^2 surface area per well) coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, 2 mM glutamine and D-glucose (total 21 mM). Cultures were maintained in humidified 5% CO_2 incubators at 37^0C with experiments performed on cultures DIV 13-15.

^{14}C-Cystine Uptake

Radiolabeled cystine uptake into spinal cord slices was measured by exposure to slice buffer containing ^{14}C-cystine (0.15μCi/ml) for 30 minutes at 37°C, in the presence or absence of 300μM of the system x_c- inhibitor sulfasalazine (SSZ). The slices were then washed for 2 minutes in ice cold slice buffer and dissolved in 250μL 1% SDS. A 100μL aliquot was removed and added to scintillation fluid for counting. Another 100μL aliquot was removed to measure cellular protein levels using the BCA assay. ^{14}C-cystine uptake values were normalized to cellular protein levels.

Radiolabeled cystine uptake in spinal cord glial cultures was performed as previously described, with modifications (Liu et al., 2009). Cultures were exposed to media with or without 100μM iron sulfate for 24 hours. After the drug exposure cultures were washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025μCi/mL, 200nM total cystine) for 20 minutes. Following ^{14}C-cystine exposure, cultures were washed with ice cold HEPES buffered saline solution and

dissolved in 250µl warm sodium dodecyl sulfate (0.1%). An aliquot (200µl) was removed and added to scintillation fluid for counting. A 30µL aliquot was removed to measure cellular protein levels using the BCA assay. ^{14}C-cystine uptake values were normalized to cellular protein levels.

Analysis of Glutamate Release

To measure glutamate released from spinal cord slices, slices were exposed to slice buffer in the presence or absence of 100µM cystine for 30 minutes at 37°C. The slices were then removed and dissolved in 250µL of 1% SDS. A 100µL aliquot was removed for the BCA protein assay. Also, the media the slices were bathed in was collected and analyzed for glutamate by HPLC (Agilent 1100), using a Hypersile-ODS reverse phase column, and ultraviolet detection at a 254nm wavelength, as previously described (Rush et al., 2010). Briefly, 200µL of media was derivatized with 100µL of phenylisothiocyanate (PITC), methanol, triethylamine (TEA) and dried under vacuum. Samples were then reconstituted in solvent consisting of 0.14M sodium acetate, 0.05% TEA, 6% acetonitrile, and brought to a pH of 6.4 with glacial acetic acid. The solvent was also used as the mobile phase, with the column being washed in 60% acetonitrile and 40% water between each sample run. Media glutamate concentrations were calculated by normalizing to glutamate standards ranging from 1-100µM. Glutamate values were then normalized to cellular protein levels.

^{3}H-D-Aspartate Uptake

To assess EAAT function, uptake of radiolabeled D-aspartate into spinal cord slices was measured. Slices were exposed to slice buffer containing ^{3}H-D-aspartate (0.25µCi/ml) for 30 minutes in the presence or absence of 100µM of the general EAAT

inhibitor DL-*threo*-β-Benzyloxyaspartic acid (TBOA). The slices were then washed for 2 minutes in ice cold slice buffer and dissolved in 250μL 1% SDS. A 100μL aliquot was removed and added to scintillation fluid for counting. Another 100μL aliquot was removed to measure cellular protein levels using the BCA assay. ^{3}H-D-aspartate uptake values were normalized to cellular protein levels.

EAAT function was also assessed in spinal cord glial cultures using radiolabeled D-aspartate. Cultures were exposed to media with or without 100μM iron citrate for 24 hours. Cultures were then washed with HEPES buffered saline solution and immediately exposed to ^{3}H-D-asparate (0.25μCi/ml) for 20 min. Following ^{3}H-D-aspartate exposure, cultures were washed, dissolved, and scintillation counted as for ^{14}C-cystine studies. A 30μL aliquot was removed to measure cellular protein levels using the BCA assay. ^{3}H-D-asparate uptake values were normalized to cellular protein levels.

Assay of neuronal death

Cell death was assessed in spinal cord glia cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after exposure to varying concentrations of iron citrate. Control LDH levels were subtracted from insult LDH values, and results normalized to 100% cell death caused by 10 μM A23187. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Lobner, 2000; Koh and Choi, 1987).

l2',7'-dichlorofluorescein (DCF) assay of oxidative stress

Oxidative stress was assayed by measuring DCF oxidation using a fluorescent plate reader following a modification of a previous method (Wang and Joseph, 1999;

Lobner et al., 2007). Briefly, cultures were exposed to 100μM iron citrate for the indicated time, after which they were exposed to 5-(and –6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (10μM). The carboxy-H_2DCFDA is de-esterified within cells to form a free acid that can then be oxidized to the fluorescent 2'7'-dichlorofluorescein (DCF). After a 1-hour exposure to carboxy-H_2DCFDA, cultures were washed 3 times with culture media lacking serum and dissolved in DMSO. A 200μL aliquot was taken and fluorescence was measured using a Fluoroskan Ascent fluorescence plate reader (ThermoLabsystems). The excitation filter was set at 485 nm and emission filter at 538 nm. A 30μL aliquot was removed to measure cellular protein levels using the BCA assay. Background fluorescence (no carboxy-H_2DCFDA added) was subtracted and the results normalized to cellular protein levels.

Monochlorobimane (MCB) assay of cellular reduced GSH

Cellular GSH levels were measured by MCB fluorescence. MCB forms a fluorescent compound when it reacts with GSH through a reaction catalyzed by glutathione-S-transferase. As previously described (Pauly et al., 2011) with modification, cultures were exposed to 100μM iron citrate for the indicated time, after which they were exposed to MCB (10μM). After 30 minutes the cultures were washed with ice cold HBBSS and dissolved in DMSO. A 200μL aliquot was taken and excited at a wavelength of 355 nm and emission measured at a wavelength of 460 nm using a Thermo Labsystems Fluoroskan microplate reader. A 30μL aliquot was removed to measure cellular protein levels using the BCA assay. Background (no MCB added) was subtracted and the results normalized to cellular protein levels.

Statistical analysis

Differences between test groups were examined for statistical significance by means of a t-test and one-way ANOVA followed by the Bonferroni post-hoc analysis, with a p-value <0.05 being considered significant.

Results

We first tested whether cystine uptake was altered in spinal cord slices in mice prepared from SOD1-G93A compared to nontransgenic littermate mice. Cystine uptake was the same in 55 day old mice, but in 70 day old mice there was significantly higher cystine uptake in the SOD1-G93A mice. This difference disappeared in the 100 day old mice (Figure 2.1). At all three of these time points mice were presymptomatic (neurological score of 0). Cystine uptake was also measured in 130 day old mice, which were early symptomatic (each with a neurological score of 1). Similar to the 100 day old mice, there was no significant difference in cystine uptake in the 130 day old mice.

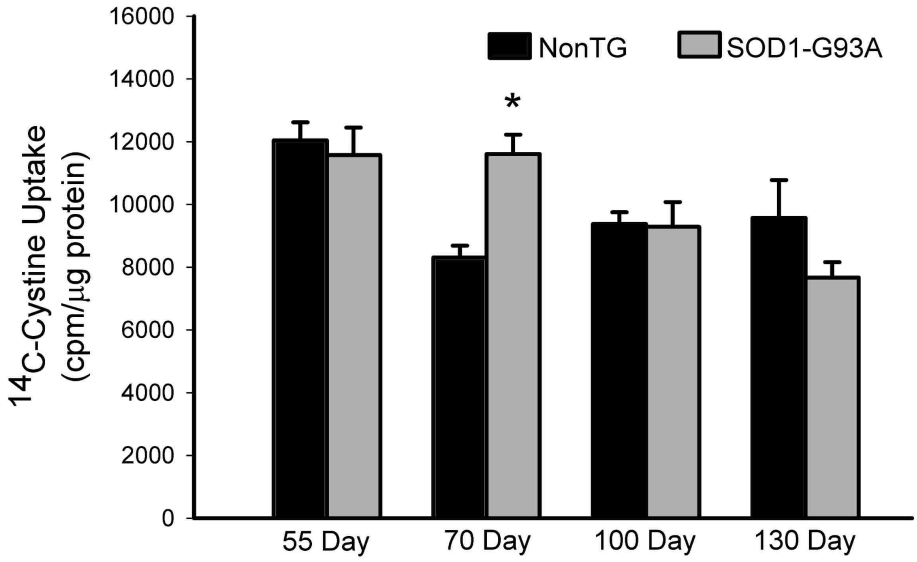

Figure 2.1 ^{14}C-cystine uptake in spinal cord slices of SOD1-G93A mice is significantly increased compared to nontransgenic (NonTG) mice at 70 days, but not at 55, 100, or 130 days of age. Bars show the counts per minute (cpm) of radiolabeled cystine per μg protein (mean ± s.e.m, n=10-16; 2-5 animals were used for each experimental group) during a 30 minute uptake assay in spinal cord slices. * indicates a significant difference within an age group, t-test ($p < 0.05$).

There are multiple mechanisms by which cystine may enter cells, to determine if the change in uptake was due to increased system x_c^- activity, we tested the inhibitor of this system, sulfasalazine (SSZ). SSZ blocked about 30% of cystine uptake in nontransgenic mice, but completely blocked the increased cystine uptake in 70 day old SOD1-G93A mice (Figure 2.2A). Graphing only the SSZ dependent cystine uptake showed that it was this component that was elevated in the SOD1-G93A mice (Figure 2.2B).

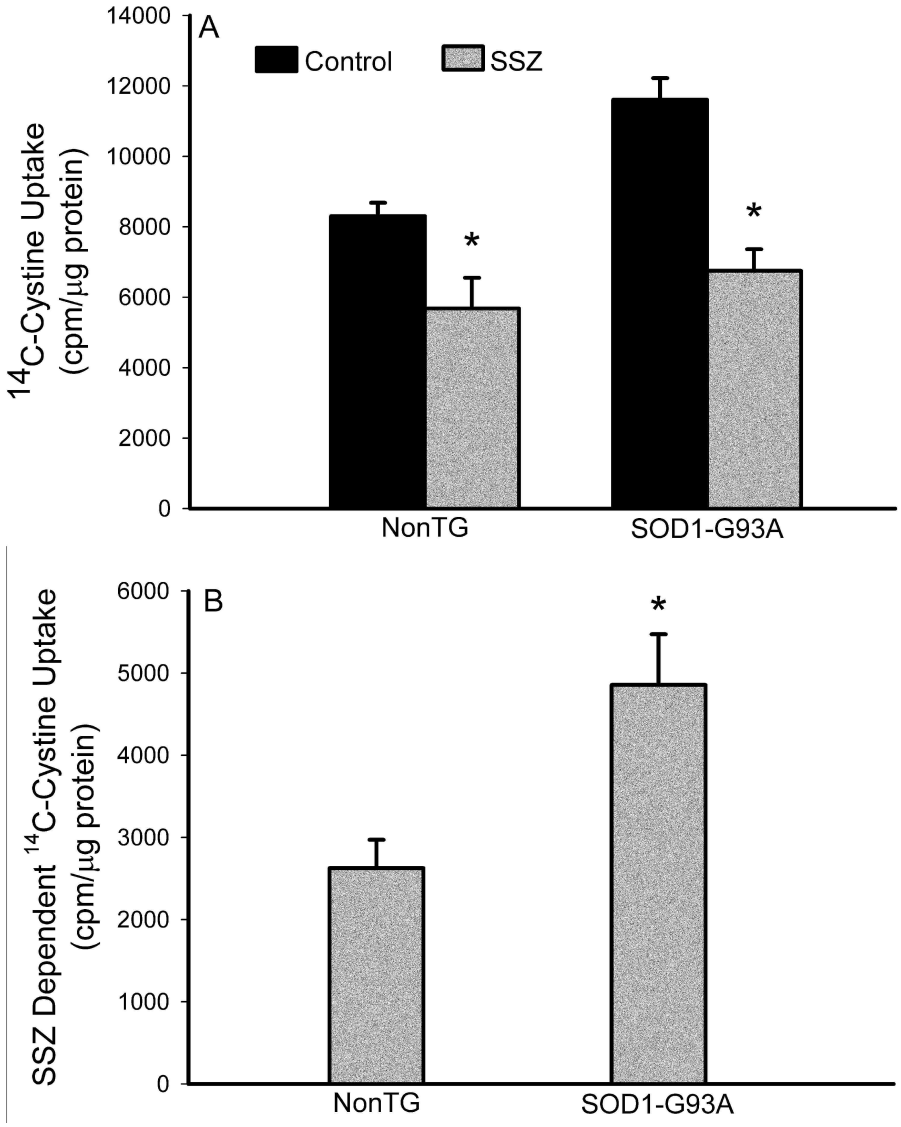

Figure 2.2 The increased ^{14}C-cystine uptake in spinal cord slices of SOD1-G93A mice is through system x_c-. A) At 70 days of age, ^{14}C-cystine uptake into spinal cord slices is significantly blocked by the system x_c- inhibitor, sulfasalazine (SSZ) in both SOD1-G93A and NonTG mice. B) The amount of cystine uptake through system x_c- is significantly higher at 70 days in SOD1-G93A mice compared to NonTG mice. Bars show the cpm of radiolabeled cystine per µg protein (mean ± s.e.m, n=16-20; 3-4 animals were used for each experimental group) during a 30 minute uptake assay in the presence or absence of 300µm SSZ. * indicates a significant difference from the NonTG mice. # indicates a significant difference within a group, t-test ($p < 0.05$).

Since each time system x_c- transports one cystine molecule into the cell it also transports one glutamate molecule out of the cell, it would also be expected to increase

glutamate release in the slices from 70 day old SOD1-G93A mice. Under conditions with no added extracellular cystine, the glutamate accumulation was slightly elevated in slices from SOD1-G93A mice. However, with 100μM cystine present in the media, there was significantly greater glutamate accumulation in the media from SOD1-G93A mouse slices (Figure 2.3).

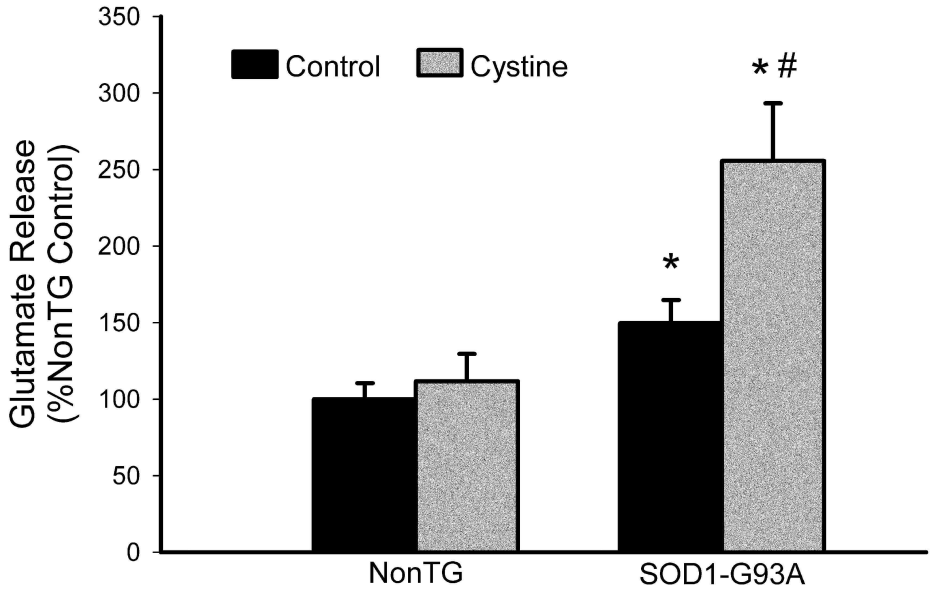

Figure 2.3 70 day old SOD1-G93A mice have higher glutamate release from spinal cord slices than NonTG mice; this release can be driven by cystine. Bars show the extracellular glutamate levels per μg protein (mean ± s.e.m, n=4-6; 2-3 animals were used for each experimental group) released from spinal cord slices during a 30 minute release assay in the presence or absence of 100μM cystine. * indicates a significant difference from the NonTG mice. # indicates a significant difference within a group, t-test ($p < 0.05$).

It is well known that glutamate uptake is decreased in both ALS patients and SOD1-G93A mice. However, glutamate uptake in spinal cord slices has not previously been tested in any ALS model. We found that glutamate uptake, measured by ^{3}H-D-aspartate uptake, was not changed in either the presymptomatic 55, 70, or 100 day old SOD1-G93A mice or in the early symptomatic 130 day old mice (Figure 2.4). To

determine whether the D-aspartate uptake was actually measuring transport by EAATs we tested the effect of the general EAAT inhibitor TBOA. TBOA blocked about 80% of ^{3}H-D-aspartate uptake in both nontransgenic and SOD1-G93A 70 day old mice (Figure 2.5A) and there was no difference in the amount of TBOA dependent uptake (Figure 2.5B).

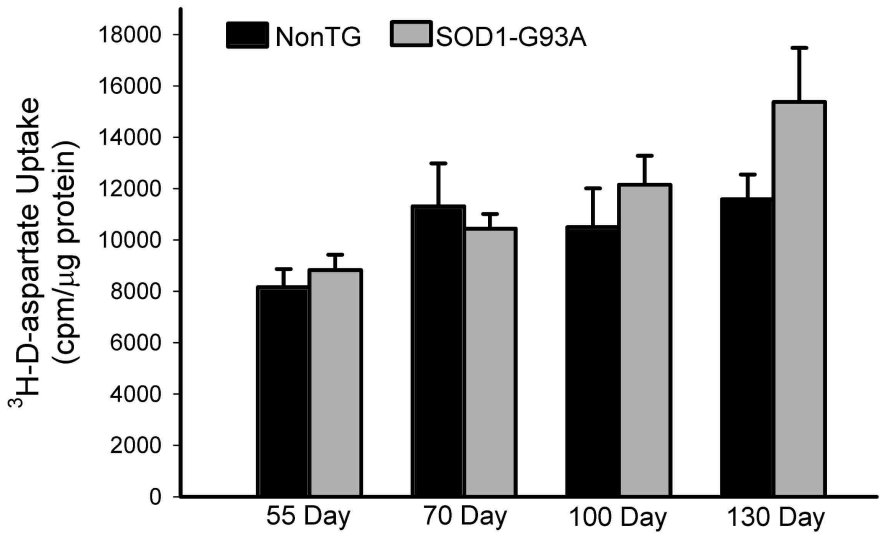

Figure 2.4 There is no significant difference in ^{3}H-D-aspartate uptake between NonTG and SOD1-G93A mice at 55, 70, 100, or 130 days of age (t-test performed). Bars show the cpm of radiolabeled D-aspartate per μg protein (mean ± s.e.m, n=11-16; 2-5 animals were used for each experimental group) during a 30 minute uptake assay in spinal cord slices.

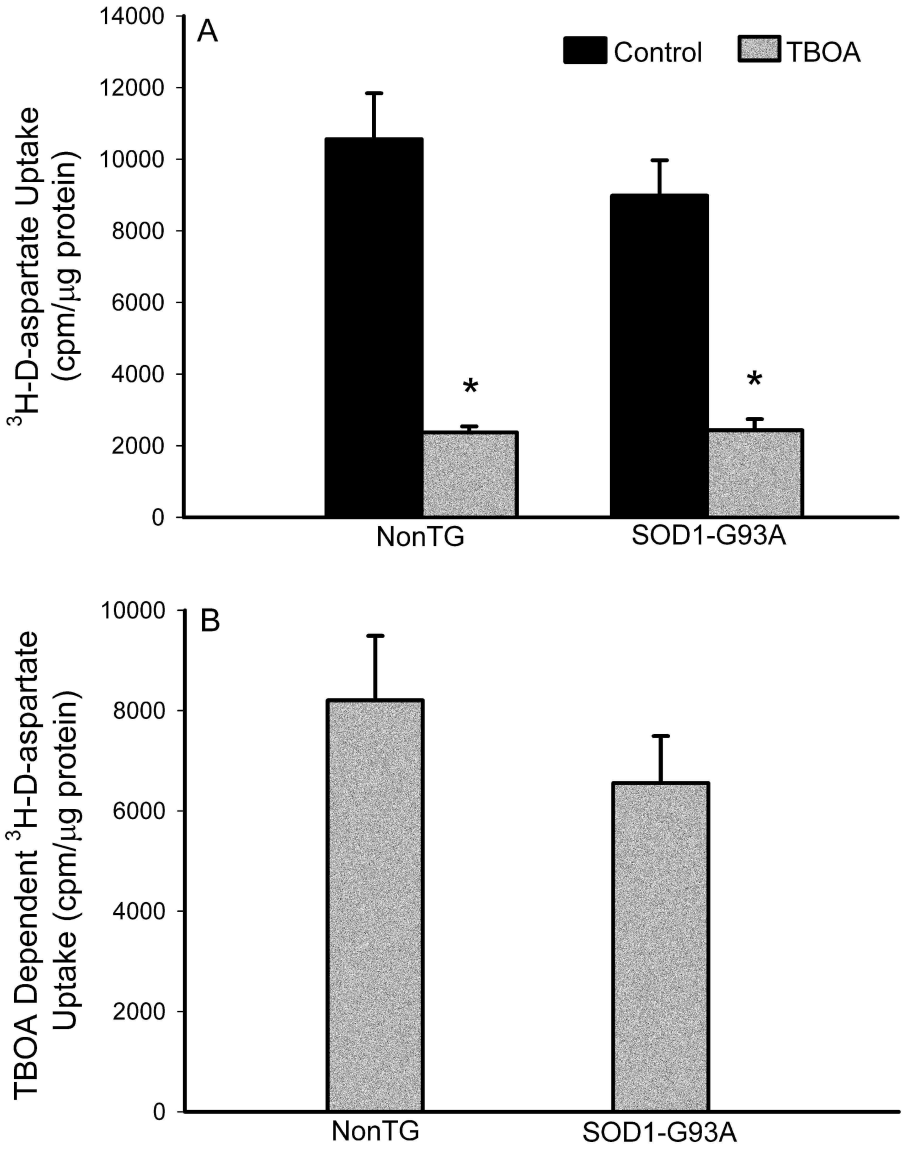

Figure 2.5 ^{3}H-D-aspartate uptake through EAATs is not significantly different between NonTG and SOD1-G93A mice. A) At 70 days of age, ^{3}H-D-aspartate uptake into spinal cord slices is significantly blocked by the excitatory amino acid transporter (EAAT) inhibitor, DL-*threo*-β-Benzyloxyaspartic acid (TBOA) in both NonTG and SOD1-G93A mice. B) The amount of D-aspartate uptake mediated by EAATs is not significantly different at 70 days in SOD1-G93A mice compared to NonTG mice. Bars show the cpm of radiolabeled D-aspartate per μg protein (mean ± s.e.m, n=8-11; 3-4 animals were used for each experimental group) taken up during a 30 minute uptake assay in the presence or absence of 100μm TBOA. * indicates a significant difference, t-test ($p < 0.05$).

Astrocytes carrying the SOD1-G93A mutation have been shown to promote motor neuron degeneration (Cassina et al., 2008; Fritz et al., 2013; Papadeas et al.,

2011). Since system x_c- is mainly found on astrocytes (Fritz et al., 2013) and has the potential to lead to excitotoxicity through increased glutamate release, we wanted to further characterize system x_c- and its regulation in SOD1-G93A astrocyte-enriched glial spinal cord cells. First, we tested whether cystine uptake was altered compared to nontransgenic littermates. Cystine uptake was the same under control conditions (Figure 2.6). In order to see if cystine uptake changed in response to oxidative stress we challenged the cells with 100μM iron citrate (Fe). As expected, cystine uptake increased significantly. Interestingly, the increase in SOD1-G93A mice was significantly lower than in nontransgenic littermates. Based upon what we observed in the spinal cord slices from SOD1-G93A mice, these results were the opposite of what we hypothesized.

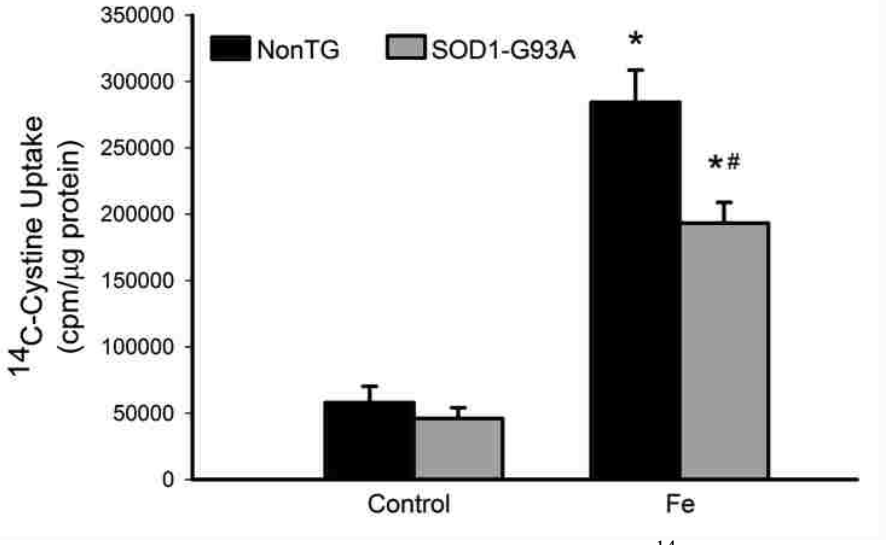

Figure 2.6 24-hour application of 100μM iron (Fe) increases ^{14}C-cystine uptake more in NonTG glial cells than in SOD1-G93A glial cells. Bars show the cpm of radiolabeled ^{14}C-cystine per μg protein (mean ± s.e.m, n=8-11) taken up during a 20 minute uptake assay. * indicates a significant difference from control; # indicates a significant difference from NonTG, t-test ($p < 0.05$).

To better understand what may be causing the difference in cystine uptake with iron application seen between cultured SOD1-G93A spinal cord cells and nontransgenic

littermates, GSH levels were measured in the presence and absence of 100μM Fe using MCB, which fluoresces when it reacts with GSH. SOD1-G93A mice did not have altered GSH levels under control conditions and 24-hour application of Fe significantly decreased GSH levels in both SOD1-G93A mice and nontransgenic littermates (Figure 2.7).

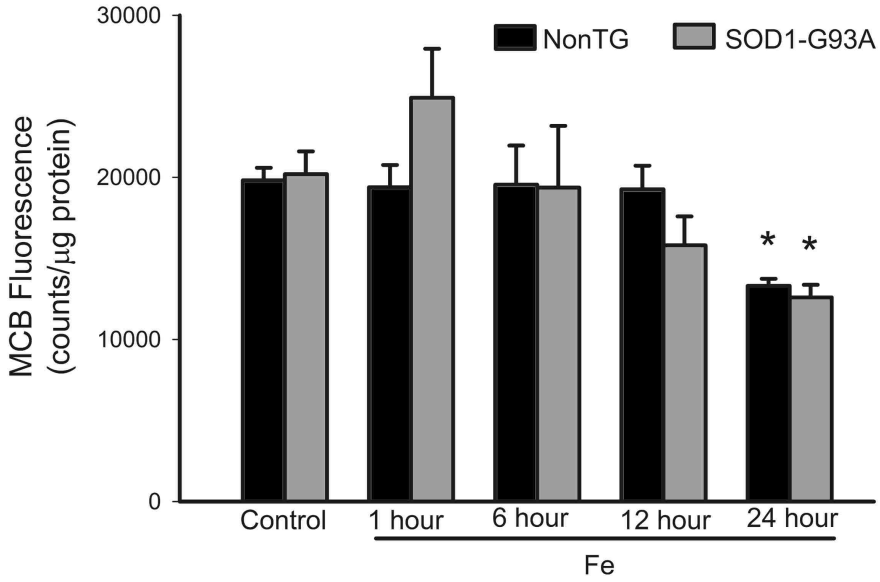

Figure 2.7 100μM Fe causes a similar time-dependent decrease in cellular glutathione levels in SOD1-G93A and NonTG glial cells. Cultures were exposed to 100μM Fe for 1, 6, 12, or 24 hours, after which cellular reduced glutathione was determined by MCB fluorescence. Bars show MCB fluorescence normalized to cellular protein levels (mean ± s.e.m, n=8-16). * indicates significant difference from control, one-way ANOVA ($p < 0.05$).

Another potential mechanism that may be causing the difference in cystine uptake is the level of oxidative stress generated by iron. To assess this cellular oxidative stress was measured with the l2',7'-dichlorofluorescein (DCF), which fluoresces when oxidized. Under control conditions, oxidative stress levels were the same; however, after a 24-hour application of 100μM Fe, oxidative stress increased significantly more in the nontransgenic littermate cultures than in SOD1-G93A cultures (Figure 2.8).

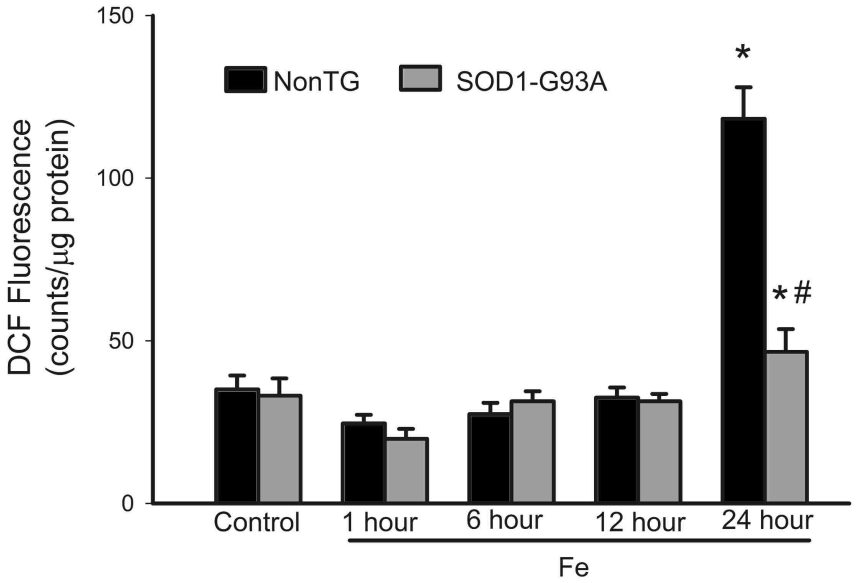

Figure 2.8 100µM Fe causes greater increase in cellular oxidative stress as measured by DCF fluorescence after 24-hour treatment in NonTG glial cells than in SOD1-G93A glial cells. Bars show DCF fluorescence normalized to cellular protein levels (mean ± s.e.m, n=8-16). * indicates significant difference from control, one-way ANOVA ($p < 0.05$); # indicates significant difference from NonTg littermate at the same timepoint, t-test ($p < 0.05$).

Since a large difference in oxidative stress induced by iron was observed, we wanted to see if iron application had different effects on glial toxicity. 100µM Fe, which is the concentration at which cystine uptake is altered, was not significantly toxic to SOD1-G93A or nontransgenic glial cells. Surprisingly, even though oxidative stress was higher in nontransgenic glial cells, a higher concentration of Fe (300µM) was more toxic to SOD1-G93A glial cells (Figure 2.9).

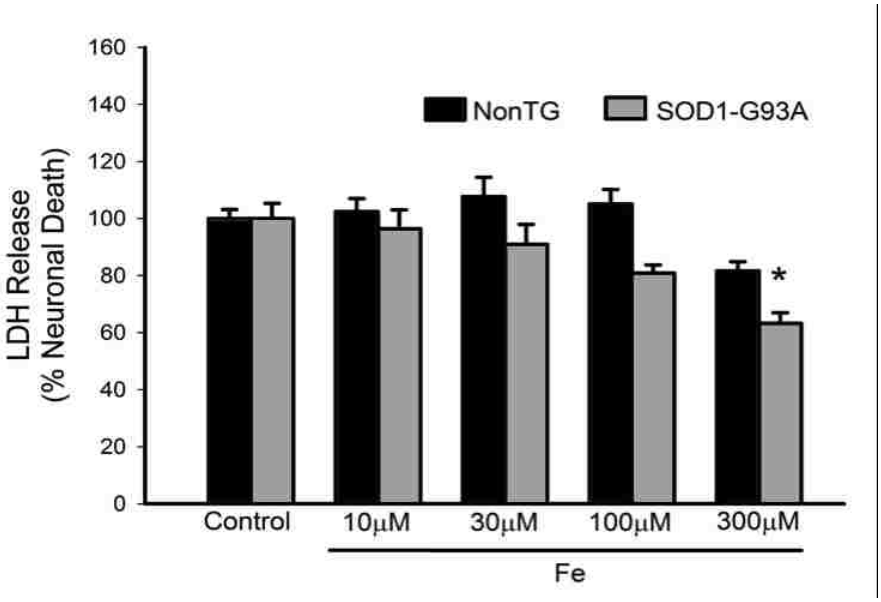

Figure 2.9 Greater toxicity is observed in SOD1-G93A cells treated with Fe than in NonTG cells. Concentration response curve for 24 hr exposure to varying concentrations of Fe on LDH release in primary glial cultures. Results are expressed as mean ± s.e.m (n=8-16). * indicates significant difference from untreated control, one-way ANOVA ($p < 0.05$).

Since we observed a difference in cystine uptake in the spinal cord glial cultures compared to uptake in spinal cord slices, we wanted to determine if ^{3}H-D-aspartate might also be different. In contrast to what was observed in slices, cultured SOD1-G93A spinal cord cells have significantly lower ^{3}H-D-aspartate uptake under control conditions. Interestingly, when challenged with 100μM Fe, ^{3}H-D-aspartate uptake does not decrease any further in SOD1-G93A mice (Figure 2.10).

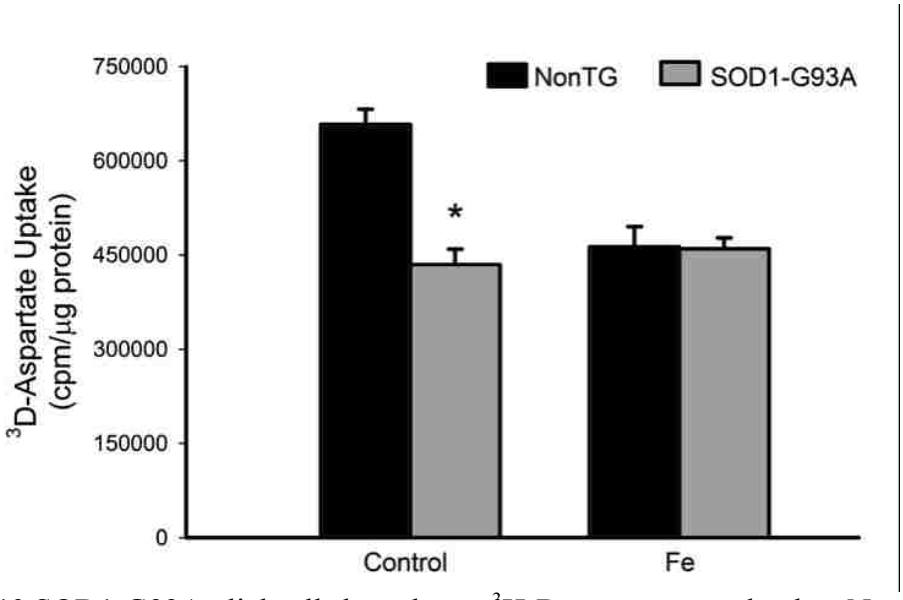

Figure 2.10 SOD1-G93A glial cells have lower ^{3}H-D-aspartate uptake than NonTG cells under control conditions. 24-hour application of 100µM Fe decreases ^{3}H-D-aspartate uptake in NonTG cells, but not in SOD1-G93A cells. Bars show the cpm of radiolabeled ^{14}C-cystine per µg protein (mean ± s.e.m, n=8-11) taken up during a 20 minute uptake assay. * indicates a significant difference from NonTG control, t-test ($p < 0.05$).

Discussion

The present study is the first to evaluate the function of system x_c^- in the spinal cord of any model of ALS. The role of system x_c^- in neuronal death is complex; it has potentially damaging effects due to its ability to release glutamate while also having potentially protective effects due to its ability to take up cystine, leading to enhanced antioxidant capabilities. The relative contribution of excitotoxicity and oxidative stress in ALS is not certain, and the potential for crosstalk between the two mechanisms exists. For example, neuronal death induced by excitotoxicity can involve oxidative stress (Dugan et al., 1996) and oxidative stress can induce glutamate release (Globus et al., 1995). A previous study by Liu et al. (1998) showed that in the spinal cord of SOD1-G93A transgenic mice there is a significant increase in oxidative stress beginning at day 60. In this study we observed a significant enhancement in cystine uptake in spinal cord

slices from presymptomatic, 70 day old SOD1-G93A transgenic mice compared to nontransgenic mice. This increase was not present at day 55 and was no longer present at day 100. We also did not observe a difference in cystine uptake in 130 day old, early symptomatic SOD1-G93A transgenic mice compared to their nontransgenic littermates. The enhanced uptake in the 70 day SOD1-G93A was significantly blocked by sulfasalazine (SSZ), the system x_c^- inhibitor, suggesting that the increase in cystine uptake was through system x_c^-.

There was also increased cystine driven glutamate release in the spinal cord slices of 70 day old SOD1-G93A mice. These findings suggest the possibility that the oxidative environment found in the spinal cord of SOD1-G93A transgenic mice may upregulate system x_c^-. This is a potential protective mechanism as it leads to increased cystine uptake and glutathione synthesis. However, it will also cause increased glutamate release which can induce excitotoxicity. To date the only FDA approved drug for the treatment of ALS is the anti-excitotoxic compound, riluzole (Miller et al., 2002). The high sensitivity of motor neurons to excitotoxicity is likely due to their expression of high numbers of calcium permeable AMPA receptors (Carriedo et al., 1996) and low expression of calcium binding proteins (Alexianu et al., 1994). Therefore, it is possible that the combination of oxidative stress and the glutamate released by system x_c^-, along with other potential factors, causes excitotoxicity in these mice.

The results in the SOD1-G93A astrocytes-enriched glial cells yield different results than those observed in slices. In culture, no differences in radiolabeled cystine uptake under control conditions were observed. Surprisingly, however, applying iron caused a significantly larger increase in system x_c^- function in the nontransgenic cells

than in the SOD1-G93A cells. Iron caused similar levels of GSH depletion in both SOD1-G93A cells and nontransgenic cells. However, the DCF assay revealed that iron caused a significantly larger increase in oxidative stress levels in nontransgenic cells, which may explain the larger increase in system x_c^- function observed in nontransgenic cells.

The SOD1-G93A mutation makes motor neurons more vulnerable to oxidative stress (Bridges et al., 2012a; Papadeas et al., 2011) In the current study we observe that the SOD1-G93A mutation also makes glial cells more vulnerable. 300μM Fe caused toxicity in the SOD1-G93A cells; however, 300μM Fe did not cause toxicity in nontransgenic cells, even though we observe higher levels of oxidative stress in these cells. Therefore, while SOD1-G93A cells do appear to be more sensitive, it does not appear to be due to increased oxidative stress. This finding is in agreement with a study that found transfecting astrocytes with SOD1-G93A did not cause an increase in oxidative stress (Boillée et al., 2006) . The increased toxicity in the SOD1-G93A glial cells is not due to depleted GSH levels either, but rather through a mechanism we were unable to detect.

It is interesting that system x_c^- function appears to decrease with age in nontransgenic mice. To our knowledge, this is the first study measuring system x_c^- activity over a range of ages. Decreased system x_c^- function with age could potentially be a contributing factor to the increased levels of oxidative stress observed with age (Liochev, 2013).

Previous studies have shown decreased expression of EAAT2 in ALS animal models. Decreased EAAT2 expression was found beginning at 14 weeks (98 days) in

SOD1-G93A transgenic rats (Howland et al., 2002) and mice (Bendotti et al., 2001), although another study involving these mice did not show a decrease until 35 weeks of age (Warita et al., 2002). Also, studies of functional glutamate uptake using synaptosomal preparations from SOD1-G93A transgenic mice did not indicate a significant decrease in glutamate uptake until mice were 150 days old (Canton et al., 1998). A potential mechanism for the decreased glutamate uptake observed in ALS is that caspase 3 is able to cleave and inactivate EAAT2 (Boston-Howes et al., 2006). A cleaved form of EAAT2 is found in spinal cords of SOD1-G93A mice (Boston-Howes et al., 2006) and caspase 3 is highly activated in these mice at the time point of ALS-like symptoms (Pasinelli et al., 2000). Since caspase 3 is activated in cells undergoing apoptosis, these findings suggest that cell death may be triggered before the inactivation of EAAT2.

In the current study we did not observe a significant decrease in radiolabeled D-aspartate uptake in spinal cord slices from SOD1-G93A transgenic mice at 55, 70, 100, or 130 days of age. D-aspartate uptake was significantly blocked in both 70 day old SOD1-G93A transgenic mice and nontransgenic mice by the EAAT inhibitor TBOA, showing that the uptake of D-aspartate was through EAATs. However, the amount of D-aspartate uptake blocked by TBOA was not significantly different between SOD1-G93A transgenic mice or nontransgenic littermate mice, suggesting that there was no change in EAAT function in SOD1-G93A transgenic mice at this age. Taken together, our results that system x_c^- mediated glutamate release is increased long before any potential decrease in glutamate uptake, suggest the possibility that this glutamate release may contribute to early excitotoxicity in SOD1-G93A mice.

However, the radiolabeled D-aspartate uptake results in cultured spinal cord glial cells are quite different than what was observed in the spinal cord slices. In glial cell cultures from SOD1-G93A mice there is a significant decrease in EAAT function in control conditions. This would suggest that very early on these cells have an impaired ability to handle glutamate, which could cause the increased levels of extracellular glutamate and contribute to excitotoxicity seen in ALS patients. The reason why iron application does not decrease EAAT function in SOD1-G93A cells further is unknown.

There have been a number of studies testing the effects of using N-acetylcysteine (NAC) to drive system x_c- as a potential treatment for ALS. NAC prevents increased oxidative stress and mitochondrial dysfunction in human neuroblastoma cells (SH-SY5Y) expressing the SOD1-G93A mutation (Beretta et al., 2003). In animal models of ALS the results have been mixed. In the SOD1-G93A mouse, one study found that NAC prolonged survival and delayed onset of motor impairment (Andreassen et al., 2000), while in another study it did not alter survival or disease onset (Jaarsma et al., 1998). There is one well controlled study using NAC in human ALS patients. In this randomized, double-blind, controlled trial it was found that NAC provided a small, non-significant increase in survival, with no evidence of reduction in disease progression (Louwerse et al., 1995). Interestingly, in subgroups of patients, those with disease of the limbs onset showed increased survival, while those with bulbar onset had decreased survival. This type of variability in effects may be expected given the dual actions of driving system x_c-. Supporting this view, riluzole provides greater survival promoting benefit in ALS patients with bulbar symptoms (Bensimon et al., 1994; Zoccolella et al., 2007), suggesting that excitotoxicity plays a greater role in these patients, which would

be consistent with NAC having a negative effect in these patients. It is possible that increased system x_c^- function may be particularly pronounced in these patients in which case further driving system x_c^- could lead to excitotoxicity that overwhelms the benefit of increased cystine uptake.

The data presented from spinal cord slices show there is enhanced system x_c^- function occurring at 70 days of age in the SOD1-G93A mouse, possibly due to the oxidative stress observed in these mice. This increased activity could be a protective mechanism in that the increased cystine uptake would maintain glutathione levels even under conditions of oxidative stress. Previously, it was found that in the spinal cord of SOD1-G93A mice reduced glutathione levels were decreased at 110 days of age, but not at 45 or 80 days (Chi et al., 2007). Interestingly, this study also found that oxidized glutathione was increased at both 80 and 110 days of age. These results suggest that glutathione production is increased at the 80 day timepoint, but high levels of oxidative stress are causing the formation of increased oxidized glutathione. These results are consistent with the elevated system x_c^- activity we found in 70 day old SOD1-G93A mice being induced by oxidative stress and that upregulation acts as a mechanism to maintain glutathione levels. However, the increased function of system x_c^- will also lead to increased glutamate release, which may contribute to the eventual neurological deficits.

The studies done in spinal cord glial cells seem to conflict with the results we see from spinal cord slices. The major difference in the ages of the mice used for these two sets of experiments is likey a factor in these conflicting results. Spinal cord slice experiments were performed in mice between the ages of 55 and 130 days old, while glial cultures were made from postnatal 1-3 day old pups. Major changes occur in the SOD1-

G93A mice as they age that cannot be replicated in culture. These are not the only discrepancies seen between *in vitro* and *in vivo* work done in SOD1-G93A mice. Tortarolo et al. (2004) saw a decrease in GLT-1 expression and function in primary astrocytes only four days after they are transfected with the SOD1-G93A gene. However, *in vivo* decreases in GLT-1 expression and function are not seen in the SOD1-G93A mouse until at least the symptomatic stages of the disease (Tortarolo et al., 2004) . The discrepancies seen between these studies highlight the differences between work done *in vitro* and *in vivo*. While *in vitro* studies are useful to determine mechanisms, the limitations of work done *in vitro* must be taken into consideration when translating them *in vivo*. With this in mind, further studies will be required to determine the net effects of system x_c- activity in the SOD1-G93A mouse model and in human ALS patients.

CHAPTER III

REGULATION OF SYSTEM X_C^- BY PHARMACOLOGICAL MANIPULATION OF CELLULAR THIOLS

Abstract

The cystine/glutamate exchanger (system x_c^-) mediates the transport of cystine into the cell in exchange for glutamate. By releasing glutamate, system x_c^- can potentially cause excitotoxicity. However, through providing cystine to the cell it regulates the levels of cellular glutathione (GSH), the main endogenous intracellular antioxidant, and may protect cells against oxidative stress. We tested two different compounds that deplete primary cortical cultures containing both neurons and astrocytes of intracellular GSH, L-buthionine-sulfoximine (BSO) and diethyl maleate (DEM). Both compounds caused significant, concentration and time-dependent decreases in intracellular GSH levels. However, DEM caused an increase in radiolabeled cystine uptake through system x_c^-, while unexpectedly BSO caused a decrease in uptake. The compounds caused similar low levels of neurotoxicity, while only BSO caused an increase in oxidative stress. The mechanism of GSH depletion by these two compounds is different, DEM directly conjugates to GSH, while BSO inhibits γ-glutamylcysteine synthetase, a key enzyme in GSH synthesis. As would be expected from these mechanisms of action, DEM caused a decrease in intracellular cysteine, while BSO increased cysteine levels. The results suggest that negative feedback by intracellular cysteine is a more important regulator of system x_c^- than intracellular GSH in this culture system.

Introduction

Under normal physiological conditions, the cystine/glutamate exchanger (system x_c-) mediates the transport of cystine into the cell in exchange for releasing glutamate into the extrasynaptic space. The exchange of extracellular cystine and intracellular glutamate occurs in a one to one ratio. The function of system x_c- makes it likely to play an important role in regulating neuronal survival and death. By releasing glutamate, system x_c- can increase extracellular glutamate levels and potentially cause excitotoxicity. Release of glutamate via system x_c-, from both microglia and astrocytes has been shown to enhance excitotoxicity of cortical neurons. (Qin et al., 2006; Fogal et al, 2007; Jackman et al, 2010; Liu et al., 2014) However, through providing cystine to the cell, it regulates the levels of intracellular glutathione (GSH), the main endogenous intracellular antioxidant, and in this way may protect cells against oxidative stress (Murphy et al., 1989; Shih et al., 2006).

Not only does system x_c- act to prevent oxidative stress, but it appears that such stress is an important trigger for its upregulation. Direct induction of oxidative stress has been shown to upregulate system x_c- function in a retinal ganglion cell line (Dun et al., 2006) and in retinal Muller glial cells (Mysona et al., 2009). Compounds that deplete cellular GSH levels upregulate system x_c- function in a glioma cell line (Kim et al., 2001) and in primary astrocytes (Seib et al., 2011), although there is not always a correlation between depletion of GSH and upregulation of system x_c- (Sasaki et al., 2002).

The first step in the production of GSH in the brain is believed to involve uptake of cystine, primarily into astrocytes (Kranich et al., 1998). Most of the cystine

transported into cortical astrocytes appears to be through system x_c- (Lobner, 2009). Once in the astrocytes cystine is immediately broken down by thioredoxin reductase 1 into two cysteine molecules (Arrick et al., 1985). GSH is synthesized via a two-step reaction (Beutler, 1989; Deneke and Fanburg, 1989). First, glutamate and cysteine are catalyzed to γ-glutamylcysteine by γ-glutamylcysteine synthetase. Then glutathione synthetase combines glycine with γ-glutamylcysteine forming GSH. Both glutamate and glycine are highly available in the cells, so the rate-limiting factor in the production of GSH is the levels of cysteine present in the cell (Dringen and Hirrlinger, 2003).

Glutathione can be utilized by cells to reduce reactive oxygen species; for example, superoxide produced as a byproduct of mitochondrial energy production rapidly reacts to form hydrogen peroxide which is then reduced by GSH to form glutathione-disulfide (GSSG) and water in a reaction catalyzed by glutathione peroxidase. Glutathione may also be utilized as a xenobiotic detoxicant as has been well characterized involving chemotherapeutics in cancer treatment (Salinas and Wong, 1999). That is, GSH can be directly conjugated to exogenous substrates via a disulfide bond with the free sulfhydryl groups; these reactions are directed by a class of enzymes known as glutathione-S-transferases (GSTs) (Dringen and Hirrlinger, 2003; Dringen, 2000). GSH, GSSG and the glutathione-conjugates are then exported from the cell in a glutathione-dependent manner via multi-drug resistance proteins (MRP), specifically MRP1 in the CNS (Hirrlinger and Dringen, 2005; Minich et al., 2006). GSH molecules produced by astrocytes can then be broken down in the extracellular space by glutathione reductase, amino-peptidase N, or γ-glutamyl transpeptidase. This metabolism produces the substrate cysteine, which can be taken up and utilized by neurons to produce their

own GSH (Fellin and Carmignoto, 2004; Stipursky et al., 2011; Yoshiba-Suzuki et al., 2011). In this way, neurons are dependent on astrocytes to supply the substrate for their GSH production (Dringen et al., 1999). The importance of cysteine uptake into neurons is indicated by the finding that knocking out the excitatory amino acid transporter-3 (EAAT3) greatly reduces neuronal cysteine uptake and intracellular GSH levels, resulting in decreased viability of hippocampal neurons against hydrogen peroxide insults (Chen and Swanson, 2003; Aoyama et al., 2006).

The current studies used mixed cultures of neurons and astrocytes to be able to incorporate the important interaction between these cell types. The studies involve assessing the effects of two different approaches to depleting cellular GSH. Diethyl maleate (DEM) directly conjugates to GSH while buthionine sulfoximine (BSO) inhibits γ-glutamylcysteine synthetase preventing the production of GSH. The studies were designed to determine the effects of these different mechanisms of GSH depletion on system x_c^- function.

Materials and Methods

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). Serum was from Atlanta Biologicals (Atlanta, GA, USA). NADPH was from Applichem (Darmstadt, Germany). Radiolabeled ^{14}C-Cystine was purchased from PerkinElmer (Boston, MA, USA). DCF was from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cortical Cell Cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24-well plates (2.0 cm^2 surface area per well) coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, 2 mM glutamine and D-glucose (total 21 mM). Cultures were maintained in humidified 5% CO_2 incubators at 37^0C with experiments performed on cultures DIV 13-15. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used. Experiments were performed in media lacking serum (MS) but otherwise identical to the growth media.

Assay of neuronal death

Cell death was assessed in cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the beginning of the insult. Control LDH levels were subtracted from insult LDH values, and results normalized to 100% neuronal death caused by 500 μM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Lobner, 2000; Koh and Choi, 1987). Cultures were also examined visually following trypan blue staining.

2',7'-dichlorofluorescein (DCF) assay of oxidative stress

Oxidative stress was assayed by measuring DCF oxidation using a fluorescent plate reader following a modification of a previous method (Wang and Joseph, 1999; Lobner et al., 2007). Briefly, cultures were exposed to 100μM DEM or BSO for the indicated period of time after which they were exposed to 5-(and –6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (10 mM). The carboxy-H_2DCFDA is de-esterfied within cells to form a free acid that can then be oxidized to the fluorescent 2'7'-dichlorofluorescein (DCF). After a 30 minute exposure to carboxy-H_2DCFDA, cultures were washed 3 times with culture media lacking serum. Fluorescence was then measured using a Fluoroskan Ascent fluorescence plate reader (ThermoLabsystems). The excitation filter was set at 485 nm and emission filter at 538 nm. Background fluorescence (no carboxy-H_2DCFDA added) was subtracted and the results normalized to control conditions (carboxy-H_2DCFDA added but no DEM or BSO).

Monochlorobimane (MCB) assay of cellular reduced GSH

Cellular GSH levels were measured by MCB fluorescence. MCB forms a fluorescent compound when it reacts with GSH through a reaction catalyzed by glutathione-S-transferase (Pauly et al., 2011). Cultures were exposed to the indicated concentrations of DEM or BSO for the indicated period of time after which they were exposed to MCB (10 mM). After 30 minutes the cultures were excited at a wavelength of 355 nm and emission measured at a wavelength of 460 nm using a Thermo Labsystems Fluoroskan microplate reader. Background (no MCB added) was subtracted and the results normalized to control (MCB added but no DEM or BSO).

HPLC analysis of cellular cysteine levels

To assess cysteine concentrations, cultures were exposed to MS containing the indicated drug for 6 or 24 hours. After the indicated time, cultures were washed with balanced salt solution (BSS) and then scraped into 250µL HPLC mobile phase. Cells were collected into microcentrifuge tubes, sonicated using a probe sonicator and analyzed for protein content using the common BCA method. Once the protein content was determined, the homogenized samples were spun through a centrifugal filter and the resulting protein free sample was injected onto a Shimadzu HPLC system coupled with an electrochemical detector. Separation was obtained with a reverse phase C-18 column and an ion-pairing mobile phase (50 mM citric acid, 10 mM octane sulfonic acid, pH 2.80, 1% acetonitrile). Resulting cysteine concentrations were normalized by the protein content and values are reported as percent control.

^{14}C-Cystine Uptake

Radiolabeled cystine uptake was performed as previously described with modifications (Liu et al., 2009). Cultures were exposed to MS containing the indicated drug treatments for 40 min, 6 hrs, or 24 hrs. After the drug exposure cultures were washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025 µCi/mL, 200 nM total cystine) for 20 minutes. Following ^{14}C-cystine exposure, cultures were washed with ice cold HEPES buffered saline solution and dissolved in 250µl warm sodium dodecyl sulfate (0.1%). An aliquot (200µl) was removed and added to scintillation fluid for counting. Values were normalized to control.

Statistical Analysis

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni post-hoc analysis, with $p<0.05$ being considered significant.

Results

We set out to determine whether depleting cellular GSH alters system x_c^- activity as assessed by measuring ^{14}C-cystine uptake in mixed cortical cell cultures. We have shown previously that the large majority of ^{14}C-cystine uptake in mixed cortical cultures is mediated by system x_c^- uptake into astrocytes (Lobner, 2009). In the current studies cellular GSH levels were depleted using two compounds with different mechanisms of action. DEM directly conjugates to GSH, while BSO inhibits GSH synthesis. Varying concentrations of DEM were added to mixed cortical cultures for 40 min, 6 hr, or 24 hrs, with ^{14}C-cystine uptake measured for 20 minutes following the exposure. DEM caused a significant increase in ^{14}C-cystine uptake at all timepoints at a concentration of 100μM, and at 6 and 24 hrs at the 10μM concentration (Figure 3.1). In contrast to DEM, when cultures were exposed to BSO at the same concentrations it did not cause an increase in ^{14}C-cystine uptake at any concentration or timepoint. In fact, BSO at a concentration of 100μM caused a significant decrease in ^{14}C-cystine uptake after 6 hr treatment, while both 10 and 100μM BSO caused a decrease at 24 hrs (Figure 3.1).

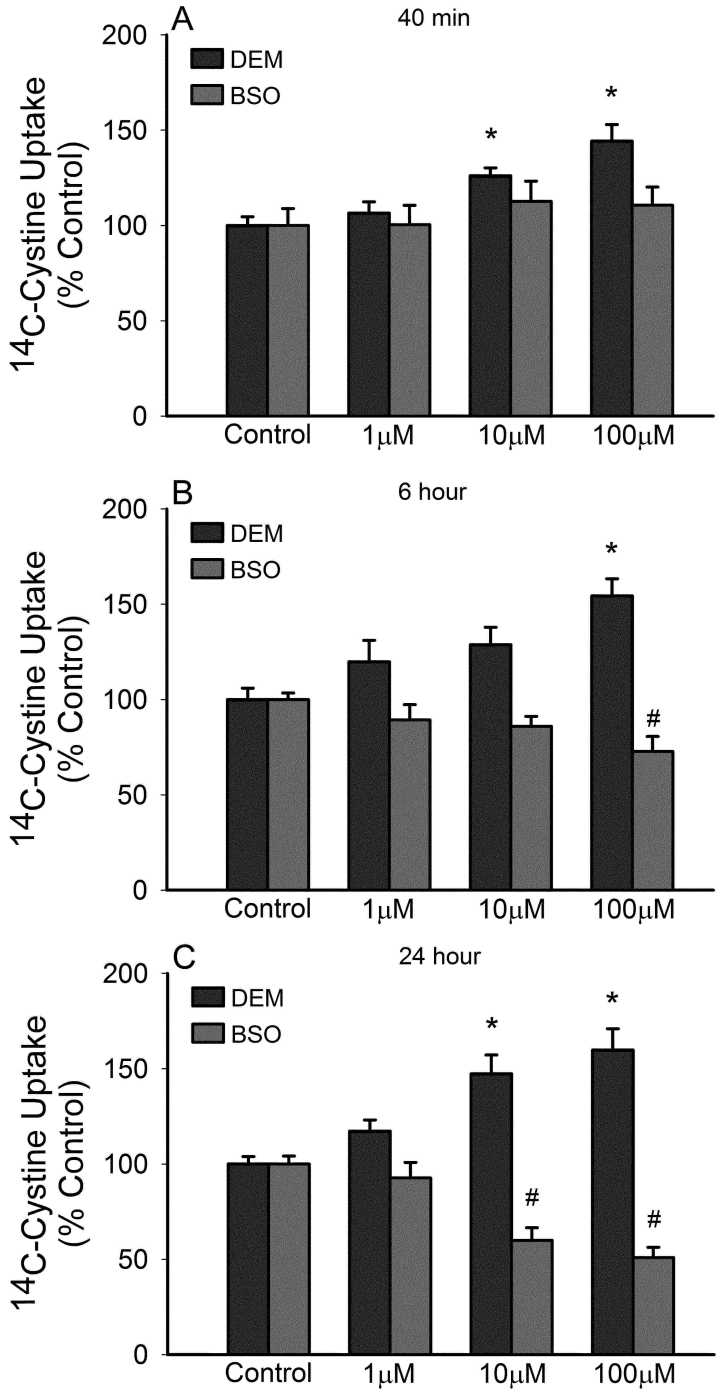

Figure 3.1 Diethyl maleate (DEM) exposure causes an increase, while buthionine sulfoximine (BSO) exposure causes a decrease, in ^{14}C-cystine uptake in mixed cortical cultures. Cultures were exposed to varying concentrations of DEM or BSO for A) 40 min, B) 6 hrs, or C) 24 hrs, after thorough washing, ^{14}C-cystine uptake was measured for 20 minutes. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control uptake. * indicates significant difference from DEM control; # indicates significant difference from BSO control, one-way ANOVA ($p < 0.05$).

To test whether the increased uptake induced by DEM treatment was mediated by system x_c-, the inhibitor of that system, sulfasalazine (SSZ), was added during the uptake period following exposure to 100μM DEM. The SSZ treatment completely blocked the increased ^{14}C-cystine uptake induced by DEM (Figure 3.2).

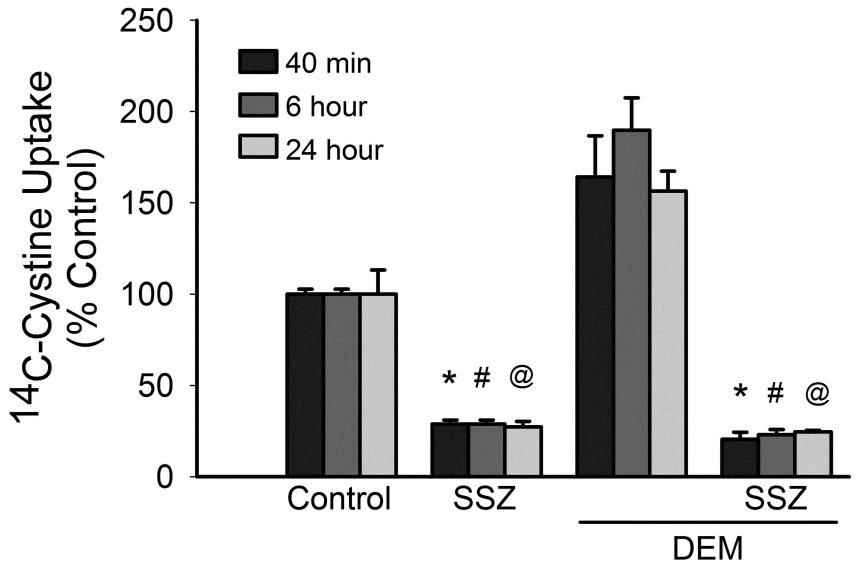

Figure 3.2 DEM induced increase in ^{14}C-cystine uptake is mediated by system x_c-. Cultures were exposed to 100μM DEM for 40 min, 6 hrs, or 24 hrs, after thorough washing, ^{14}C-cystine uptake was measured for 20 minutes with or without the system x_c- inhibitor sulfasalazine (SSZ) present. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control uptake. * indicates significant difference from 40 min control; # indicates significant difference from 6 hour control; @ indicates significant difference from 24 hour control, one-way ANOVA ($p < 0.05$).

A potential cause for altered ^{14}C-cystine uptake could be toxicity of DEM or BSO. DEM and BSO both caused a small, but significant level of neurotoxicity after 24 hours at the 100μM concentrations (Figure 3.3). Trypan blue staining indicated that the death was selective for neurons (data not shown).

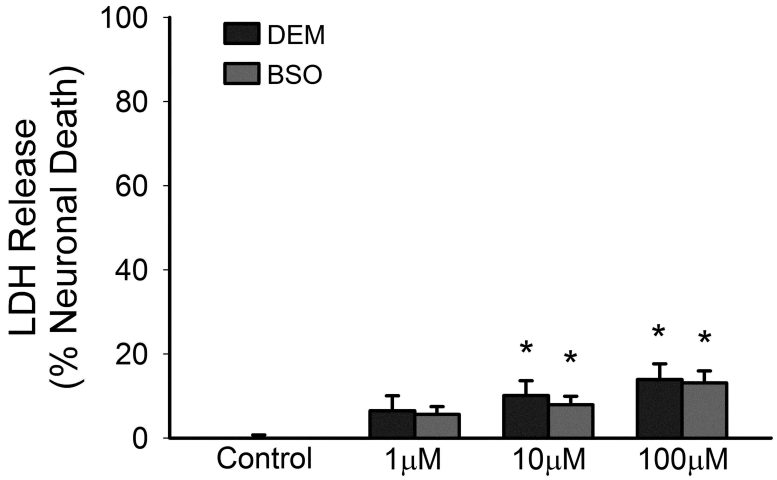

Figure 3.3 DEM and BSO cause similar low levels of neurotoxicity. Concentration response curve for 24-hour exposure to DEM and BSO on LDH release in primary cortical cultures. Results are expressed as mean ± s.e.m (n=8-16). * indicates significant difference from untreated control, one-way ANOVA ($p < 0.05$).

A potential mechanism by which DEM may have caused increased system x_c- activity is through inducing oxidative stress, which has been shown to upregulate system x_c- (Dun et al., 2006; Mysona et al., 2009). We measured cellular oxidative stress with the compound DCF, which becomes fluorescent when oxidized. Somewhat surprisingly we did not see enhanced DCF fluorescence following 100μM DEM treatment, while 100μM BSO only caused an increase following 24 hr treatment (Figure 3.4).

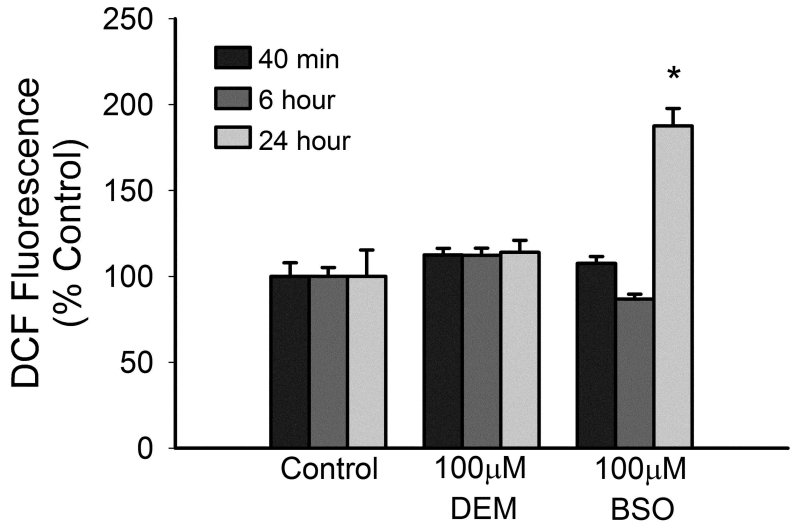

Figure 3.4 BSO, but not DEM, causes an increase in cellular oxidative stress after a 24-hour treatment. Bars show % DCF fluorescence normalized to control fluorescence (mean ± s.e.m, n=8-16). * indicates significant difference from control, one-way ANOVA ($p < 0.05$).

Another potential mechanism by which DEM may be causing increased system x_c- function is through causing decreased GSH levels. DEM and BSO treatment both caused a decrease in cellular GSH levels (Figure 3.5). There were some differences in the decrease; DEM caused a more rapid decrease in GSH, with a significant decrease at the 40 minute timepoint, while BSO did not cause a significant decrease until the 6 hr timepoint. The GSH levels with DEM treatment actually increased from the 6 hr timepoint to the 24 hr timepoint, so that at 24 hrs, BSO caused a greater decrease in GSH levels than DEM.

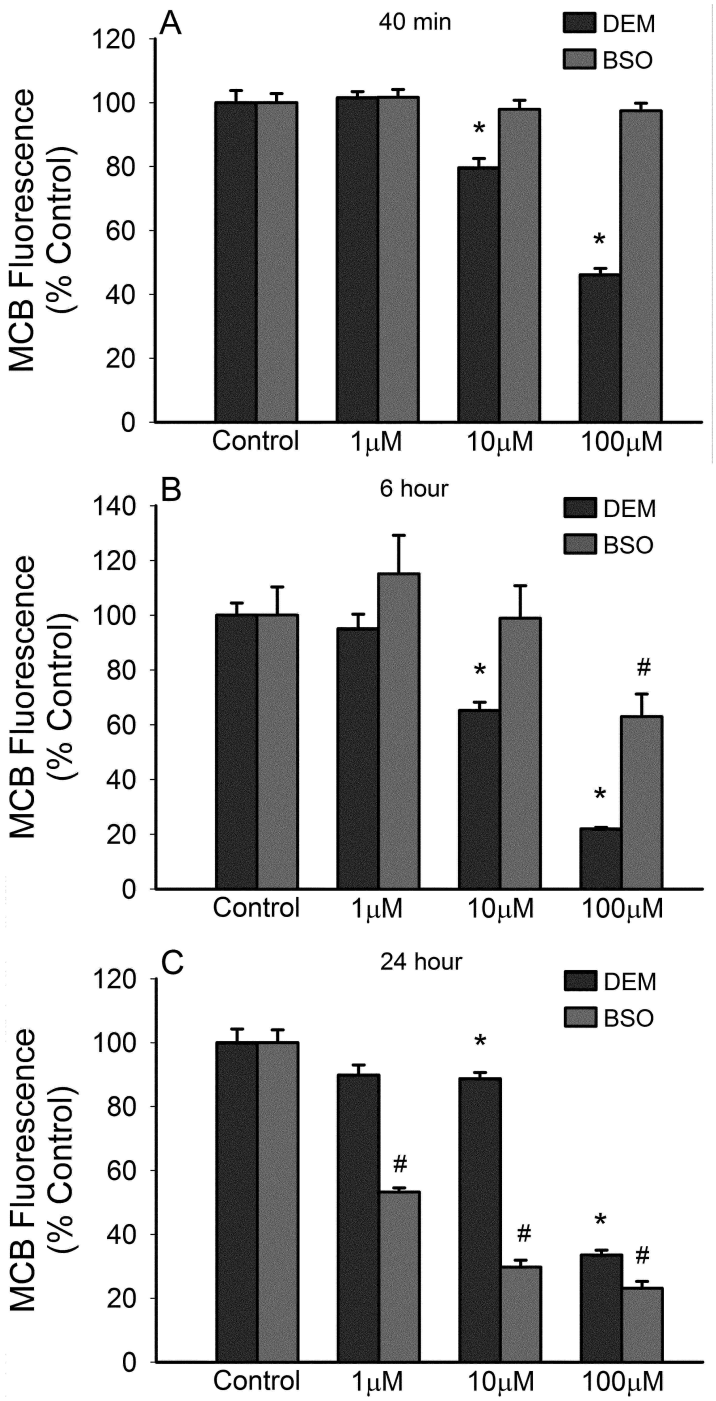

Figure 3.5 DEM and BSO cause a concentration dependent decrease in cellular glutathione levels. Cultures were exposed to varying concentrations of DEM or BSO for A) 40 min, B) 6 hrs, or C) 24 hrs, after which cellular reduced glutathione was determined by MCB fluorescence. Bars show % MCB fluorescence normalized to control fluorescence (mean ± s.e.m, n=8-16). * indicates significant difference from DEM control; # indicates significant difference from BSO control, one-way ANOVA ($p < 0.05$).

While DEM and BSO both act to decrease GSH levels they do so by different mechanisms suggesting the possibility that they may alter cellular cysteine levels differently. We found that 100μM DEM caused a significant decrease in cellular cysteine levels after 6 hr treatment with the effect disappearing at 24 hours, while 100μM BSO caused a significant increase in cellular cysteine after 6 and 24 hr treatment (Figure 3.6).

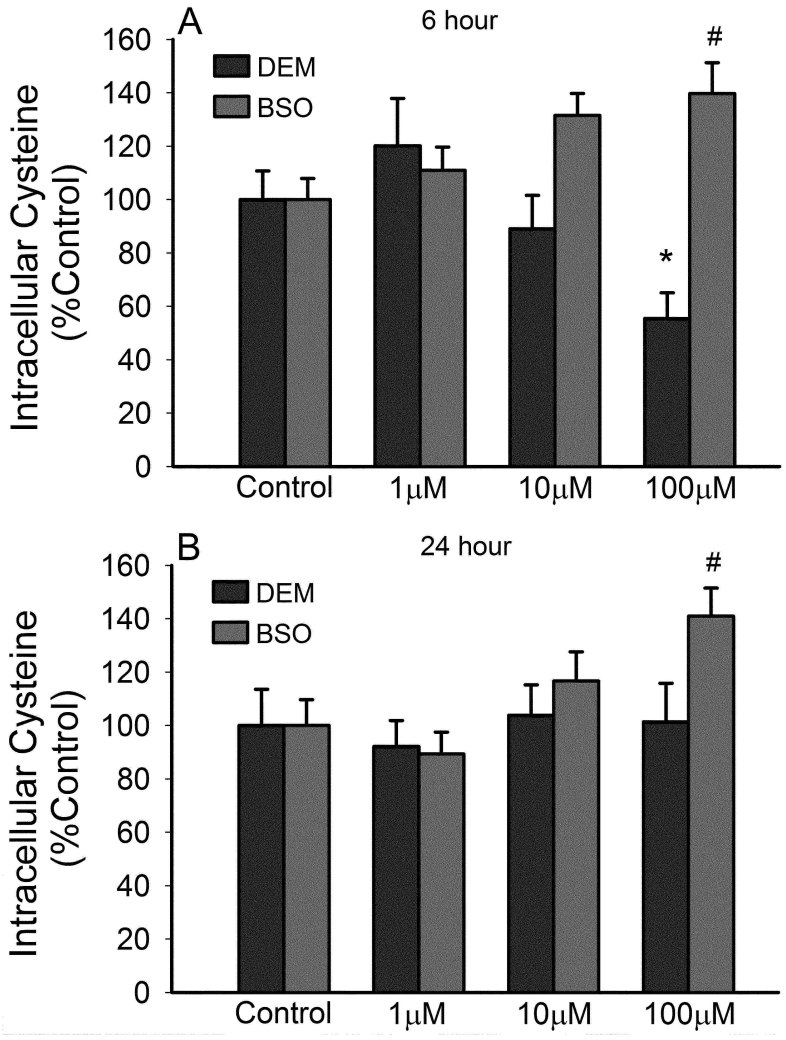

Figure 3.6 DEM causes an early decrease in cellular cysteine levels, while BSO causes an early and late increase in cellular cysteine levels. Cultures were exposed to varying concentrations of DEM or BSO for A) 6 hrs or B) 24 hrs, after which cellular cysteine levels were determined by HPLC. Bars show % cellular cysteine normalized to control (mean ± s.e.m, n=8). * indicates significant difference from DEM control; # indicates significant difference from BSO control, one-way ANOVA ($p < 0.05$).

Discussion

The regulation of system x_c- function is proving to be complicated, likely because of its varied functions. Upregulation of system x_c- by oxidative stress has been well established and this regulation is mechanistically understandable considering that system x_c- is important for cystine uptake and therefore GSH production. However, system x_c- function has also been shown to be upregulated by a diverse array of compounds including IL-1b (Fogal et al., 2007), erythropoietin (Sims et al., 2010), FGF-2 (Liu et al., 2012), IGF-1 (Pauly et al., 2011; Yang and Yee, 2014), TGF-b (Pauly et al., 2011), and PACAP (Resch et al., 2014). While system x_c- function has been shown to be decreased by dexamethasone (Piani and Fontana, 1994), regulation by these diverse compounds may reflect the importance of system x_c- in regulating not only oxidative stress but also extracellular glutamate. For example, it has been shown that cocaine addiction is associated with impaired system x_c- function leading to decreased activation of presynaptic group II mGluRs leading to increased synaptic release of glutamate (Baker et al., 2002; Baker et al., 2003). It is likely that the regulation of system x_c- by these compounds reflects the importance of regulating both intracellular GSH and extracellular glutamate. Another factor to consider is that there is an interaction between system x_c- and excitatory amino acid transporters (EAATs) (Lewerenz et al., 2009). The possibility that altered EAAT function could change glutamate concentrations and in this way change system x_c- function exists.

The goal of the current study was to examine in more detail the regulation of system x_c- by agents that deplete GSH. The previous thinking about such agents is that they upregulated system x_c- by depletion of cellular GSH, and either this depletion

directly stimulated system x_c-, or the resulting increased oxidative stress caused the upregulation. Our results indicate another potential mechanism of regulation. In our studies, simple depletion of cellular GSH did not appear to be the trigger for upregulation of system x_c-. While there were some differences in the time course and concentration dependence of the effects of DEM and BSO treatment on GSH levels, they both caused depletion of GSH and yet they had opposite effects on system x_c- function. These results are difficult to explain by the different time courses in effects on GSH levels. If BSO caused no effect on system x_c- function this could potentially be explained by its slower depletion of GSH, but the fact that it actually caused a decrease in system x_c- activity seems unlikely to be explained by the slower loss of GSH with BSO treatment than with DEM treatment. Interestingly, the GSH levels following DEM treatment actually increased from the 6 hr timepoint to the 24 hr timepoint (Figure 3.5). This increase is likely due to the upregulation of system x_c- under these conditions leading to increased cystine uptake and additional substrate for GSH production.

Toxicity due to treatment with DEM or BSO could be a potential confound when assessing cystine uptake. However, both compounds caused only a small degree of neurotoxicity (10-15%) after 24 hours. This toxicity seems unlikely to play a major role in the results for three reasons. First, DEM and BSO caused similar toxicity but had opposite effects on cystine uptake. Second, the levels of cell death were small compared to the magnitude of changes in cystine uptake. Third, the cell death was selective for neurons, while most the cystine uptake in this culture system is into astrocytes (Lobner, 2009).

The result that DEM did not induce oxidative stress, as measured by DCF fluorescence, suggests that increased oxidative stress is not the mechanism by which DEM upregulates system x_c^- function, particularly since BSO did induce oxidative stress while it actually decreased system x_c^- function. However, it was somewhat surprising that DEM did not induce oxidative stress since it did decrease the levels of GSH. There are a number of possible explanations for this result. First, GSH is not the only antioxidant present in the brain. Under the conditions we were studying it is possible that decreased GSH levels would not lead to enhanced oxidative stress in the cells. Second, the DCF assay does not detect all forms of free radicals (Gomes et al., 2005) and it may be less effective in detecting mitochondria selective oxidative stress (Karlsson et al., 2010). Therefore while we cannot absolutely conclude that oxidative stress is not the trigger for upregulation of system x_c^- by DEM, the fact that BSO caused a marked increase in oxidative stress while DEM did not, and yet BSO caused a decrease in system x_c^- function, suggests that factors other than oxidative stress are more important in regulation of system x_c^- in this system.

Our results suggest that, at least for our cell culture system, cysteine is a more important regulator of system x_c^- function than GSH. DEM caused both a decrease in cysteine and GSH levels, consistent with its action to conjugate GSH leading to constant use of cellular cysteine. Therefore, either the decrease in cysteine or GSH could be responsible for the upregulation of system x_c^-. However, BSO also decreased GSH, but increased cysteine levels, consistent with its action to inhibit γ-glutamylcysteine synthetase leading to GSH depletion but buildup of cysteine, and it caused a downregulation of system x_c^- function. In this case, the most likely explanation for the

effect is that the buildup of cysteine provides negative feedback on system x_c^- function. This type of regulation makes sense physiologically. The levels of cysteine in the cell are more directly related to the uptake of cystine than they are to the levels of GSH. Glutathione levels in cells could be altered by changes in the function of γ-glutamylcysteine synthetase or glutathione synthetase, or potentially the availability of glycine or glutamate. Therefore, if system x_c^- was upregulated by GSH depletion it could be in response to conditions unrelated to the availability of cystine. Additionally, cysteine levels in the brain are 100 times lower than GSH levels (Slivka and Cohen, 1993) and therefore, rapid changes in their levels are more likely to occur than changes in GSH levels.

Our results are in contrast to those of Seib et al. (2011), who found a large increase in system x_c^- function in astrocytes after treatment with BSO. There are two major differences in the culture systems used for the studies. First, we used a mixed neuronal and astrocyte culture, while in the Seib et al. study they used a pure astrocyte culture. The possibility exists that the interaction of neurons with the astrocytes alters the how-cells-regulate system x_c^-. Second, their cultures received long-term treatment with a cell permeant form of cAMP prior to the BSO treatment. From their studies, it is clear that GSH levels can also be an important regulator of system x_c^-, but that the status of the cells likely determines the relative importance of cysteine or GSH in the regulation. We cannot exclude the possibility that GSH plays a role in regulating system x_c^- even in our culture conditions, but it appears that cysteine levels have a greater effect than GSH on system x_c^- function. The implications this finding has for the role of system x_c^- in disease conditions is not certain. Cysteine levels are not as commonly measured as GSH

levels. For example, we have found that system x_c^- function is increased at 70 days of age in the G93A-SOD1 mouse model of ALS (Albano et al., 2013), a timepoint at which GSH levels are not yet decreased (Chi et al., 2007), but intracellular cysteine levels are unknown.

In conclusion, our studies indicate that, at least under some conditions, intracellular levels of cysteine are a more important regulator of system x_c^- than intracellular levels of GSH. We did not determine the mechanism of regulation by cysteine, but the redox sensitive transcription factor Nrf2 has been shown to be the main regulator of system x_c^- (Sasaki et al., 2002; Shih et al., 2003). This finding puts system x_c^- in the context of it being one factor in the role of Nrf2 as the master regulator of the cellular response to oxidative stress (Ishii and Mann, 2014). In conclusion, studies involving assessment of levels of cysteine, GSH, and system x_c^- function during disease conditions will be required to determine the most important regulator of system x_c^- function in disease states.

CHAPTER IV

TGF-β1 INDUCED UPREGULATION OF SYSTEM X_C- IN ASTROCYTES ENHANCES NEURONAL DEATH

Abstract

System x_c-, the cystine/glutamate exchanger, mediates the transport of one cystine molecule into the cell in exchange for the release of one glutamate molecule into the extrasynaptic space. Cystine transported into the cell is converted to glutathione (GSH), the main antioxidant in the brain, which protects the cell from oxidative stress. However, the release of glutamate into the extrasynaptic space can lead to excitotoxicity. Transforming growth factor-β1 (TGF-β1) is a cytokine involved in regulating many cellular processes, including neuronal survival and death. In this study we determined whether TGF-β1 regulates system x_c- function and if system x_c- plays a role in the effects of TGF-β1 activity on neuronal survival/death. We found that TGF-β1 increased ^{14}C-cystine uptake through system x_c- in astrocyte-enriched glial cultures, which required both TGF-β1 receptors ALK5 and ALK1 and was mediated through the MAPK/ERK pathway. TGF-β1 increased xCT mRNA levels and the increased uptake was blocked by the protein synthesis inhibitor cycloheximide. Interestingly, TGF-β1 increased the export of GSH from astrocytes to be utilized by neurons, which would suggest a neuroprotective role for TGF-β1. However, in mixed cortical cultures TGF-β1 enhanced rotenone-induced toxicity, an effect mediated through AMPA receptors. Together the data suggests that the increase in system x_c- activity by TGF-β1 may have antioxidant defenses, but can also exacerbate excitotoxicity.

Introduction

Transforming growth factor-β1 (TGF-β1) is a cytokine with wide ranging effects in the CNS, including regulating cellular processes like growth and development (Böttner et al., 2000; Massagué et al., 2000), differentiation (Ishihara et al., 1994), and apoptosis (Prehn et al., 1994; Zhu et al., 2001; 2002). TGF-β1 is one of three TGF-β isoforms and is part the TGF-β superfamily, which includes multiple cytokines. While its receptors, TGF-β receptor I and II (TGF-βRI, -II), are found throughout the brain and are expressed on neurons, astrocytes, and microglia (Böttner et al., 1996; Vivien et al., 1998; Vivien and Ali, 2006), TGF-β1 expression is very low during development and in the adult brain (Flanders et al., 1991; Pelton et al., 1991; Unsicker et al., 1991). While some studies show it is mainly confined to the meninges and choroid plexus (Unsicker et al., 1991), others have found it is present in the cerebral cortex (Vivien et al., 1998). However, in response to injury and neurodegeneration TGF-β1 is highly upregulated and secreted by many different cell types (Pratt and McPherson, 1997; Flanders et al., 1998; Vivien and Ali, 2006), which makes its potential role in neuronal protection and death of interest.

TGF-β1 has been well studied in models of focal and global ischemia, where it is upregulated in the infarct and penumbral areas (Krupinski et al., 1996) and has protective effects (Prehn et al., 1993; Henrich-Noack et al., 1996; Pang et al., 2001). It is also protective against β-amyloid toxicity (Prehn et al., 1996; Ren and Flanders, 1996), MPP+ toxicity to dopaminergic neurons (Krieglstein et al., 1995), apoptosis (Prehn et al., 1994; Zhu et al., 2001; 2002) and oxidative stress mediated neuronal death (Henrich-Noack et al., 1996). Interestingly, while TGF-β1 has been shown to be neuroprotective in acute

excitotoxicity models, in chronic excitotoxicity models TGF-β1 enhances toxicity (Prehn and Kriedglstein, 1994; Prehn and Miller, 1996).

The cystine/glutamate antiporter (system x_c-) is a sodium-independent amino acid transporter found on astrocytes, which takes up one molecule of cystine into the cell in exchange for one molecule of glutamate (Bannai and Kitamura, 1980; Bannai, 1986). The cystine that is imported is quickly reduced to cysteine and used to synthesize glutathione (GSH) (Bannai and Kitamura, 1980). GSH is the main endogenous antioxidant in the brain and protects against oxidative stress; it can be released by astrocytes and used to protect neurons (Shih et al., 2003). Cysteine can also be released by astrocytes and taken up by surrounding neurons to synthesize GSH (Lewerenz et al., 2006; Wang and Cynader, 2000). In exchange for taking up one molecule of cystine, system x_c- releases one molecule of glutamate into the extrasynaptic space and is the major source of extracellular glutamate in several rodent brain regions (De Bundel et al., 2011; Massie et al., 2011; Baker et al. 2002). The glutamate released by system x_c- can activate extrasynaptic receptors and regulate synaptic function (Baker et al., 2002; Williams and Featherstone, 2014). However, high extracellular levels of glutamate released via system x_c- can also lead to excitotoxicity (Fogal et al., 2007; Hardingham and Bading, 2010; Liu et al., 2014). The dual function of system x_c- makes it likely to play an important role in regulating neuronal survival and death.

Both TGF-β1 and system x_c- are known to play a role in oxidative stress and excitotoxicity, key players in many neurodegenerative diseases. TGF-β1 and system x_c- are also upregulated in neurodegenerative diseases (Flanders et al., 1998; Vivien and Ali, 2006; Massie et al., 2015). It is our hypothesis that TGF-β1 upregulates system x_c- on

astrocytes and some of the actions of TGF-β1 may be due its effects on system x_c-. In this present study we used primary astrocyte-enriched glial cortical cultures to study the effects of TGF-β1 on system x_c- and then used primary mixed cortical cultures to investigate TGF-β1's effect on neuronal survival/death in concert with an increase in oxidative stress, a hallmark of many neurodegenerative diseases.

Materials and Methods

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). Serum was from Atlanta Biologicals (Lawrenceville, GA). TGF-β1 was from Prospec (East Brunswick, NJ) and ^{14}C-cystine was from PerkinElmer (Waltham, MA). TRIzol was obtained from Life Technologies (Grand Island, NY). The reverse transcription kit was from Promega (Madison, WI) and the PerfeCTa SYBR Green FastMix with ROX from Quanta Biosciences (Gaithersburg, MD). Primers were obtained from Integrated DNA Technologies (Coralvilla, IA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2mM glutamine and glucose (total 21 mM). Neuron-enriched cultures were prepared exactly as above with the addition of 10μM cytosine arabinoside 48 hours after plating to inhibit

glial replication. In these cultures <1% of cells are astrocytes (Dugan et al., 1995; Rush et al., 2010). Astrocyte-enriched glial cultures were prepared as described for mixed cultures except they are from cortical tissue taken from post-natal day 1-3 mice (Choi et al., 1987; Schwarts and Wilson, 1992; Rush et al., 2010). Pure astrocyte cultures were prepared from astrocyte-enriched glial cultures as described by Hamby et al. (2006). Microglia cultures were prepared by first growing astrocyte enriched glial cultures, then shaking the microglia free and plating them in media containing 10 ng/ml colony stimulating factor (CSF) (Barger and Basile, 2001). Cultures were maintained in humidified 5% CO_2 incubators at 37°C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

^{14}C-Cystine Uptake

System x_c^- mediated uptake of radiolabeled cystine was assayed as previously described with modifications (Liu et al., 2009). Astrocyte-enriched glial cultures were exposed to media lacking serum (MS) containing 100ng/mL TGF-β1 for the indicated time in humidified 5% CO_2 incubators at 37°C. In experiments where inhibitors were used, the inhibitors were added 1 hour prior to the addition of TGF-β1 and remained in the media for the duration of the incubation time. After the indicated time, cultures were washed with HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025μCi/mL) for 20 minutes. In experiments where ^{14}C-cystine uptake through system x_c^- was blocked, 300μM sulfasalazine (SSZ) was added just prior to radiolabeled cystine. Following ^{14}C-cystine exposure, all cultures were washed with HEPES buffered saline

solution and dissolved in 250µL warm sodium dodecyl sulfate (0.5%). A 200µL aliquot was removed and added to scintillation fluid for counting. Values were normalized to ^{14}C-cystine uptake in untreated controls on the same experimental plate.

Assay of neuronal death

Mixed and astrocyte-enriched glial cultures were washed with MS containing 100ng/mL TGF-β1 for 24 hours. 100nM rotenone was then added, either with or without the other inhibitors indicated for another 24 hours. Cell death was assessed in mixed and astrocyte-enriched glial cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells, in the extracellular fluid 48 hours after the beginning of the insult. Control LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 µM NMDA for mixed cultures or 100% astrocyte death caused by 20µM of the calcium ionophore A23187, added 24 hours before the assay. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner, 2000).

Monochlorobimane (MCB) assay of cellular reduced GSH

Cellular GSH levels were measured by MCB fluorescence. MCB forms a fluorescent compound when it reacts with GSH through a reaction catalyzed by glutathione-S-transferase (Pauly et al., 2011). Astrocyte-enriched glial cultures were exposed to MS containing 100ng/mL TGF-β1 for 24 hours. MCB (final concentration of 10µM) was added to each sample 30 minutes prior to the end time. A 200µL sample was taken from the media. The cells were then washed three times with cold HBBSS and dissolved with 250µL DMSO. A 200µL sample was placed into a 96-well plate.

Samples were read at 355/460 with a Fluoroskan Ascent Microplate Fluorometer (Thermo LabSystems). Results were normalized to control conditions.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Transcriptional expression of xCT in astrocyte-enriched glial cultures after stimulation with TGF-β1 was examined using RT-qPCR. Briefly, after treating cells with TGF-β1 for various time points, cells were washed 3 times with cold PBS. Total RNA was isolated with TRIzol extraction (Invitrogen; Carlsbad, CA) according to manufacturer's protocol. RNA quality was determined using spectrophotometry with a 260/280 ratio of >1.8. cDNA was synthesized using the Reverse Transcription System (Promega; Madison, WI) from 1 mg of total RNA following the manufacturer's protocol. All products were amplified on a StepOne real-time PCR system (Applied Biosystems; Carlsbad, CA) using 100 ng of cDNA, PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersburg, MD), and the following primers designed using Primer3 software: gapdh mouse forward-AAG GGC TCA TGA CCA CAG TC; gapdh mouse reverse-GGA TAC AGG GAT GAT GTT CT; xCT mouse forward- TCA CTT TTT GGA GCC CTG TC; xCT mouse reverse- ACC CAG ACT CGA ACA AAA GC. Relative quantification of xCT transcripts was analyzed via the $2^{-\Delta\Delta Ct}$ method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Differences between test groups were examined for statistical significance by means of t-test or one-way ANOVA followed by the Bonferroni correction post-hoc test, with $p<0.05$ being considered significant.

Results

We first tested the effects of TGF-β1 on ^{14}C-cystine uptake in purified cultures of astrocytes, microglia, and neurons. A 24-hour treatment with TGF-β1 caused a significant increase in ^{14}C-cystine uptake in pure astrocyte cultures, but not in microglia or pure neuronal cell cultures (Figure 4.1). The following experiments, unless otherwise noted, were performed in astrocyte-enriched glial cultures. We next tested if the TGF-β1 induced increase in ^{14}C-cystine uptake was mediated by system x_c-; addition of the system x_c- inhibitor, sulfasalazine (SSZ), blocked the TGF-β1 induced increase in ^{14}C-cystine uptake (Figure 4.2).

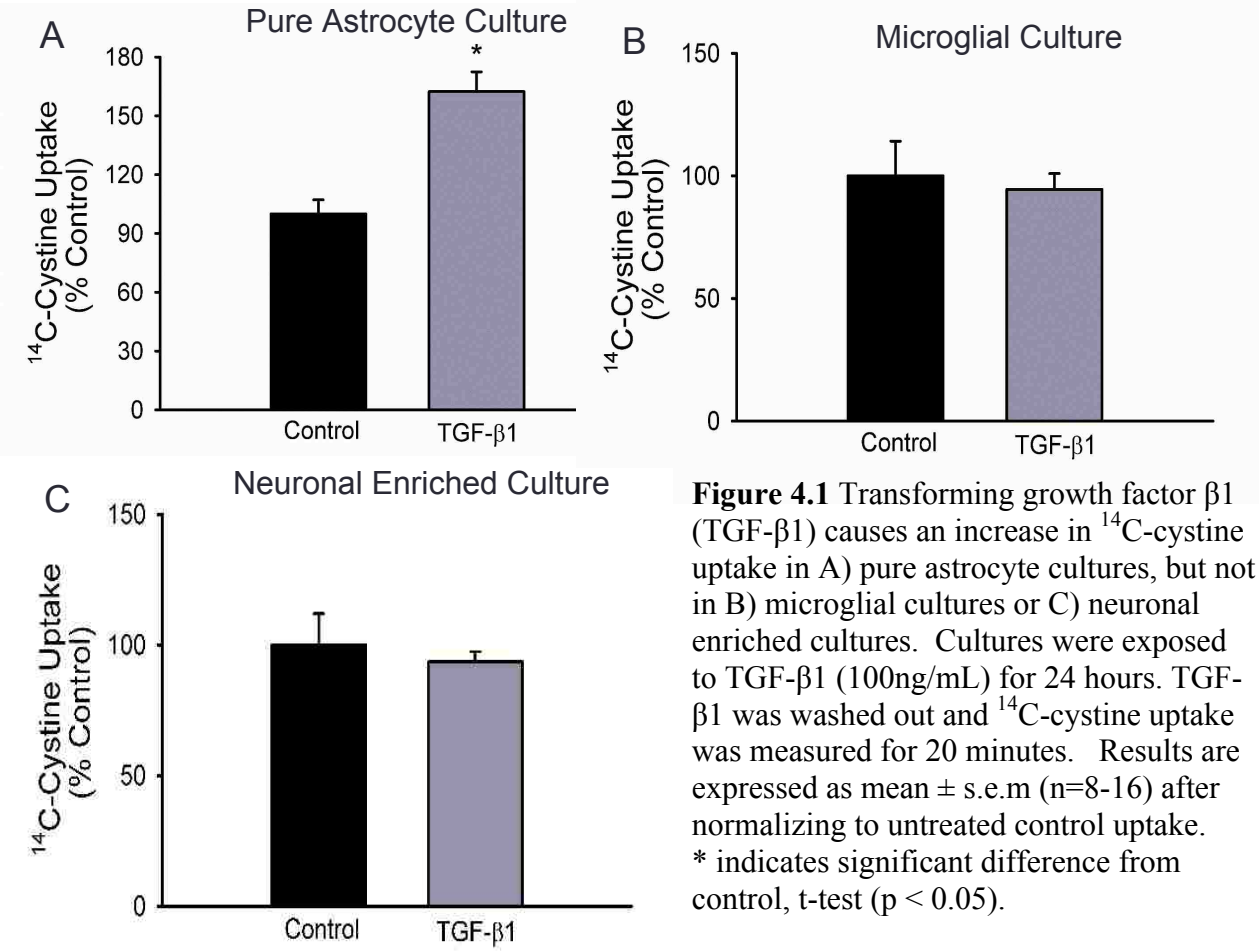

Figure 4.1 Transforming growth factor β1 (TGF-β1) causes an increase in ^{14}C-cystine uptake in A) pure astrocyte cultures, but not in B) microglial cultures or C) neuronal enriched cultures. Cultures were exposed to TGF-β1 (100ng/mL) for 24 hours. TGF-β1 was washed out and ^{14}C-cystine uptake was measured for 20 minutes. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control uptake. * indicates significant difference from control, t-test ($p < 0.05$).

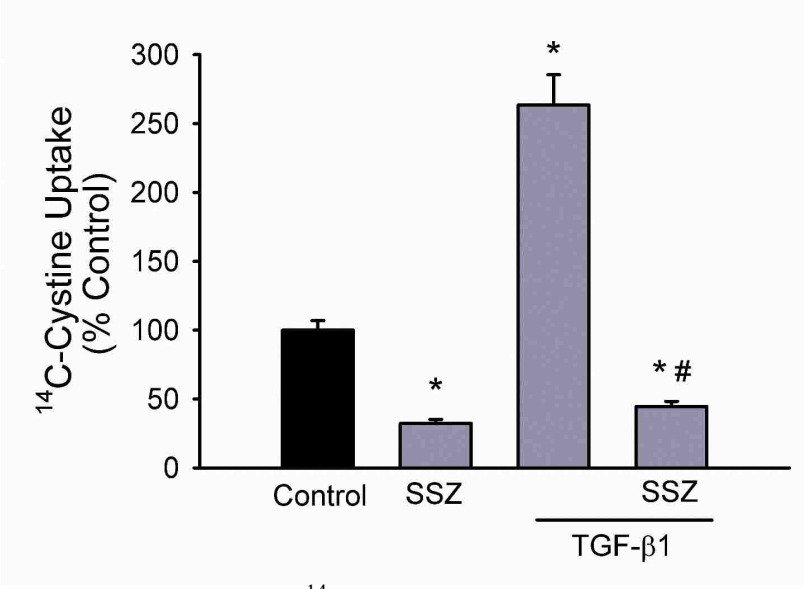

Figure 4.2 TGF-β1 increases ^{14}C-cystine uptake through system x_c-. Astrocyte enriched cultures were exposed to TGF-β1 (100ng/mL) for 24 hours and after thorough washing, ^{14}C-cystine uptake was measured for 20 minutes with or without the system x_c- inhibitor sulfasalazine (SSZ, 300mM) present. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control uptake. * indicates significant difference from control; # indicates significant difference from TGF-β1 alone, one-way ANOVA ($p < 0.05$).

The increased system x_c- mediated ^{14}C-cystine uptake could be mediated by alteration of function of already present system x_c- or increased system x_c- protein. To assess this, we first performed a time course of TGF-β1's effects on ^{14}C-cystine uptake. TGF-β1 caused no increase in ^{14}C-cystine uptake with treatment up to 12 hours (Figure 3A). The long duration exposure for increased uptake suggests new protein synthesis is required. To test this we added the protein synthesis inhibitor cycloheximide (CHX), which completely blocked the TGF-β1 induced increase, during a 24-hour treatment (Figure 3B). Since there are questions regarding antibody reliability (Massie et al., 2015) we did not perform western blotting for the functional subunit of system x_c-, xCT. However, we performed qPCR and found that 24-hour application of TGF-β1 increased xCT mRNA levels (Figure 3C).

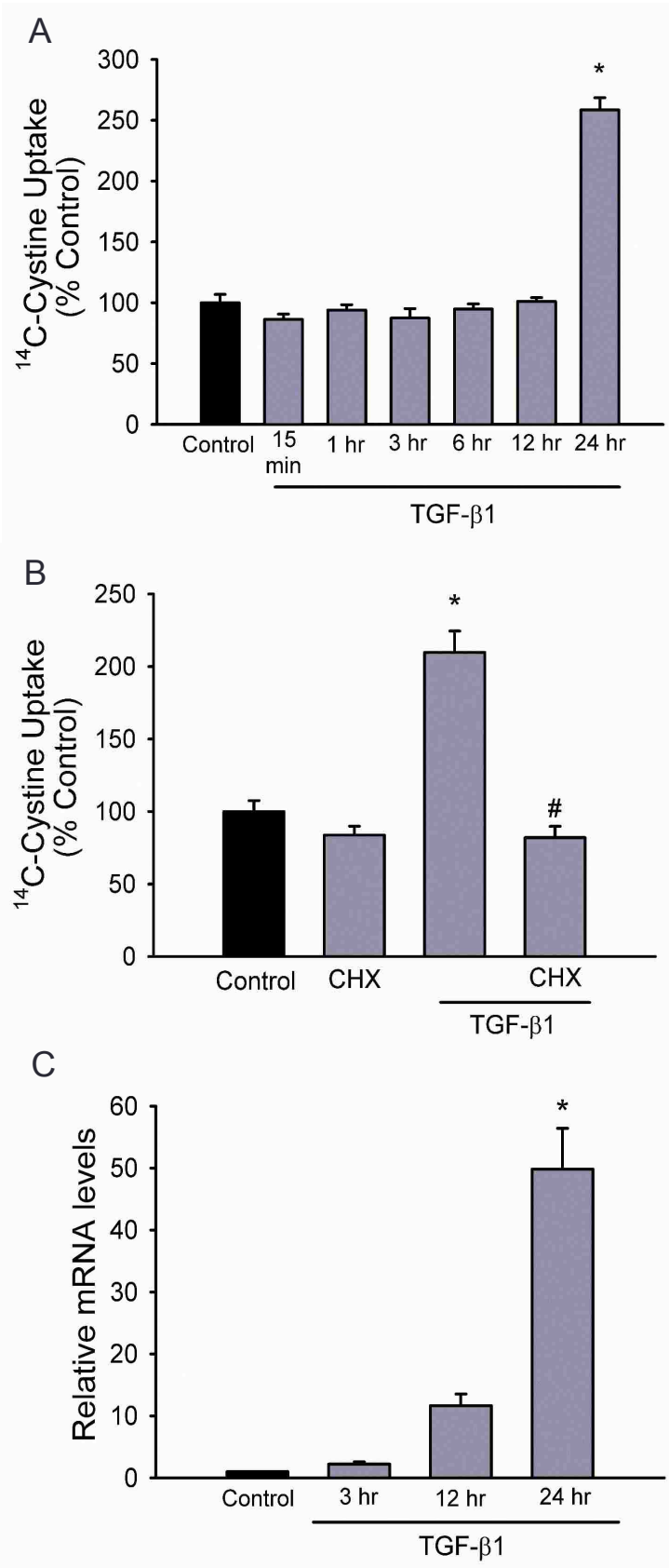

Figure 4.3 TGF-β1 induces a time and protein synthesis dependent increase in ^{14}C-cystine uptake and increases xCT mRNA expression. A) TGF-β1 (100ng/mL) was applied for various times to astrocyte-enriched cultures. Cultures were then washed and a 20 min ^{14}C-cystine uptake assay was performed. B) Cultures were exposed to TGF-β1 with or without the protein synthesis inhibitor cycloheximide (CHX, 200ng/mL) for 24 hours and after thorough washing, ^{14}C-cystine uptake was measured for 20 minutes. Results are expressed as mean±SEM (n=8-16) after normalizing to untreated control uptake. C) Astrocyte-enriched cultures were exposed to TGF-β1 for the duration indicated. RNA was extracted using TRIzol and RT-PCR was used to determine mRNA expression. Results were normalized to controls (set to 1) and presented as mean ± s.e.m (n=4-5). * indicates significant difference from control; # indicates significant difference from TGF-β1 alone, one-way ANOVA ($p < 0.05$).

TGF-β1 acts by binding to TGF-β receptor II (TGF-βRII), which then recruits and phosphorylates a second receptor, TGF-βRI. There are two TGF-βRI isoforms that TGF-β1 signals through: activin receptor-like kinase 5 (ALK5) and ALK1 (Massagué, 1998). We tested the ALK 5 inhibitor LY364947 and the ALK1 inhibitor K02288. Each of the inhibitors caused a partial decrease in the TGF-β1 enhancement of ^{14}C-cystine uptake, with application of both inhibitors causing a near complete inhibition (Figure 4.4A). TGF-β1 activates multiple intracellular pathways (Massagué, 2000). We tested the role of the MEK/ERK pathway in the upregulation of system x_c-. We found that the MEK/ERK pathway inhibitor U0126 largely blocked the effect of TGF-β1 (Figure 4.4B).

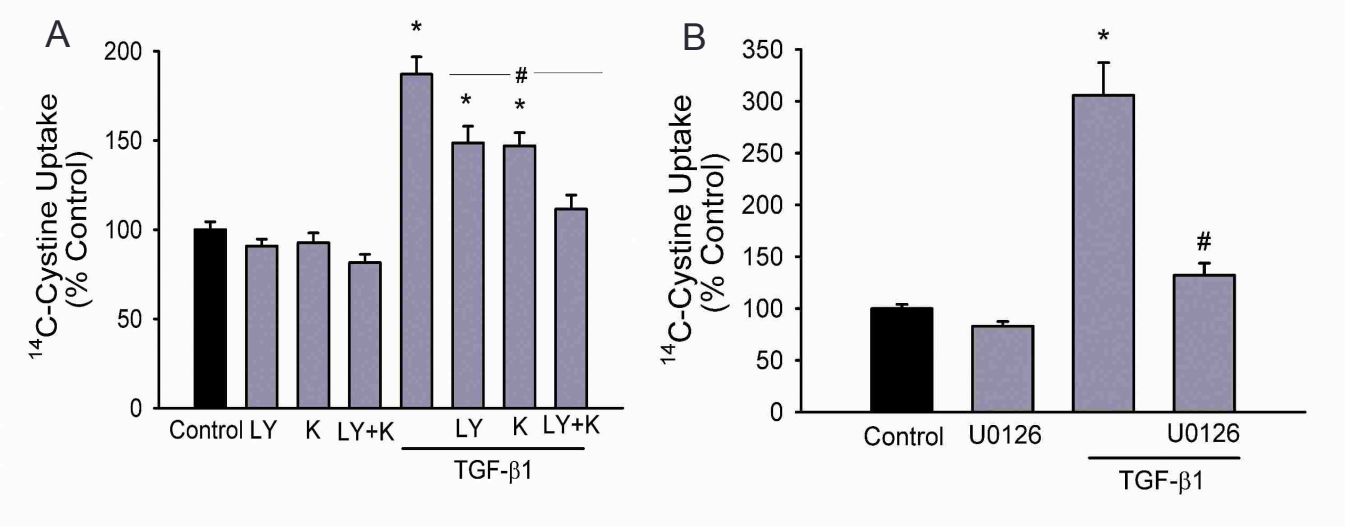

Figure 4.4 TGF-β1 induced increase in ^{14}C-cystine uptake is mediated by activation of both ALK5 and ALK1 and the MAPK/ERK pathway. A) Astrocyte-enriched cultures were treated with the ALK5 inhibitor LY364947 (LY, 10μM) and/or the ALK1 inhibitor K02288 (K, 10μM) for 1 hour prior to and during TGF-β1 (100ng/mL) exposure for 24 hours. After a thorough washing, ^{14}C-cystine uptake was measured for 20 minutes. B) Cultures were exposed to the MAPK/ERK pathway inhibitor U0126 (10μM) 1 hour prior and during exposure to TGF-β1 for 24 hours and after thorough washing, ^{14}C-cystine uptake was measured for 20 minutes. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control uptake. * indicates significant difference from control; # indicates significant difference from TGF-β1 alone, one-way ANOVA ($p < 0.05$).

The increase in cystine uptake would be expected to cause an increase in intracellular GSH. However, in astrocyte-enriched cultures 24-hour treatment with TGF-β1 actually caused a decrease in cellular GSH (Figure 4.5A). There was also a small decrease in pure neuronal cultures, but no effect in mixed cultures (Figure 4.5A). Testing the levels of GSH in the media, we found an increase in the media from astrocyte cultures, and small decrease in pure neuronal and mixed cultures (Figure 4.5B). The results suggest that TGF-β1 may be stimulating the astrocytes to release glutathione, which in turn supplies substrate for glutathione production in neurons. To test this neurons were grown on culture inserts in the presence of astrocytes; after TGF-β1 treatment cultures were separated in order to test GSH levels in each cell type individually. TGF-β1 treatment caused a decrease in cellular GSH in the astrocytes, but no change in neurons (Figure 4.6).

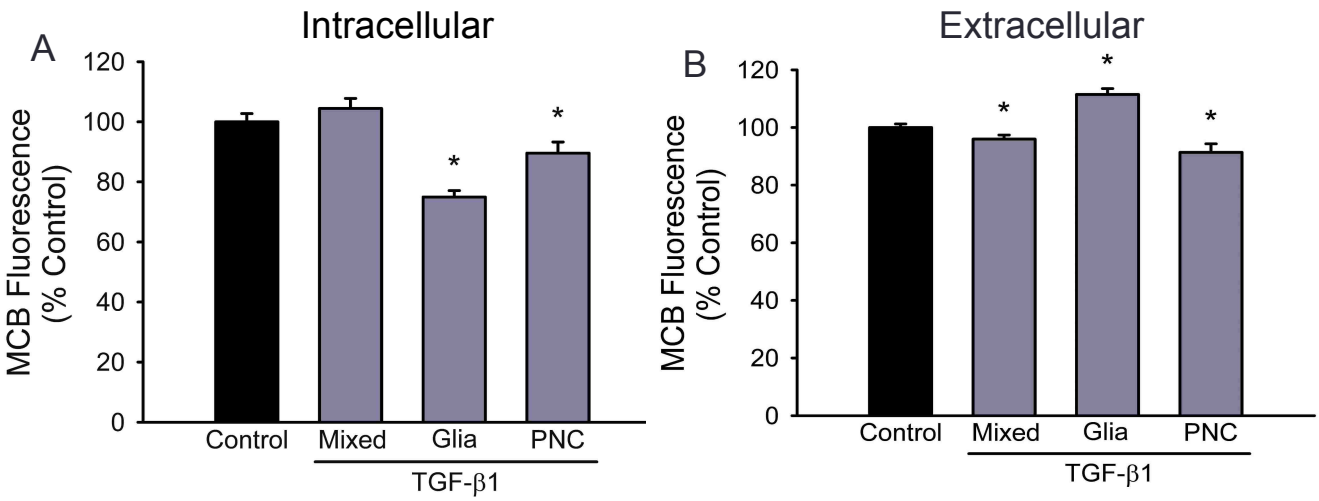

Figure 4.5 TGF-β1 decreases intracellular GSH levels in astrocyte-enriched (glia) and neuronal-enriched (PNC) cultures, but not in mixed cultures; TGF-β1 increases extracellular GSH levels in astrocyte-enriched cultures. TGF-β1 (100ng/mL) was applied to the indicated culture system for 24 hours. 30 minutes prior to the end time 1mM MCB was applied. Both the A) intracellular and B) extracellular GSH levels were measured. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control. * indicates significant difference from control, t-test ($p < 0.05$).

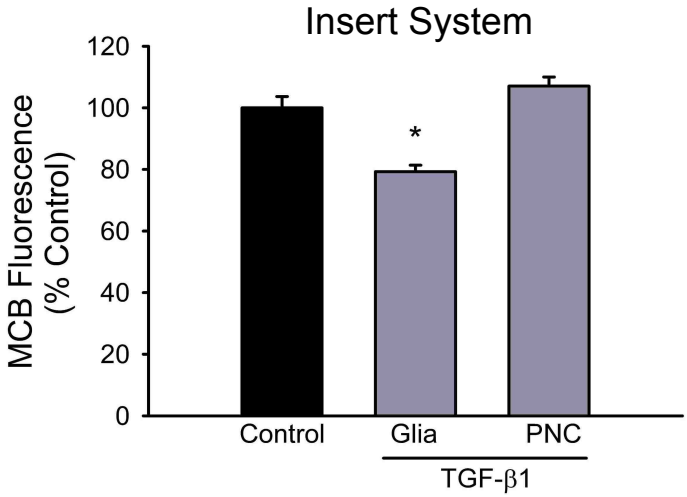

Figure 4.6 In an insert system, TGF-β1 decreases intracellular GSH levels in astrocyte-enriched (glia) cultures, but not in neuronal-enriched (PNC) cultures. TGF-β1 (100ng/mL) was applied for 24 hours to astrocytes and neurons that had been cultured together using an insert system. 30 minutes prior to the end time 1mM MCB was applied. The astrocytes and neurons were then separated so intracellular GSH levels could be measured in each cell type separately. Results are expressed as mean ± s.e.m (n=8-16) after normalizing to untreated control. * indicates significant difference from control, t-test ($p < 0.05$).

Upregulation of system x_c^- can have complicated effects on cell death due to its dual role of providing cystine for GSH production, but also releasing glutamate, which can cause excitotoxicity. We found that treatment of mixed cultures with TGF-β1 for up to 48 hours did not cause any cell death (Figure 4.7). However, when cultures were treated with TGF-β1 for 24 hours prior to and during 24-hour exposure to the mitochondrial inhibitor rotenone (100nM) the TGF-β1 treatment enhanced the toxicity of rotenone (Figure 4.7). The neuronal death induced by exposure to TGF-β1 and rotenone was attenuated by treatment with the AMPA/kainate receptor antagonist, NBQX, (Figure 4.8A) but not by the NMDA antagonist, memantine (MEM), (Fig 4.8B) or the calcium permeable AMPA receptor antagonist NASPM (Figure 4.8C). The toxicity was purely neuronal, as the same treatment did not cause LDH release in astrocyte cultures (Figure 4.9). However, TGF-β1 did enhance rotenone-induced astrocyte toxicity when higher levels of rotenone were applied (Figure 4.9).

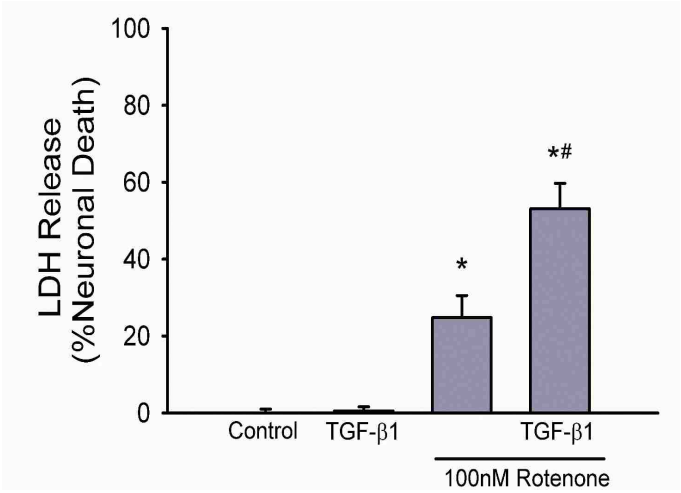

Figure 4.7 TGF-β1 enhances rotenone-induced toxicity in neurons. TGF-β1 (100ng/mL) was applied for 48 hours to mixed cortical cultures. 24 hours after TGF-β1 application, 100nM rotenone was applied. Bars show % death (mean ± s.e.m, n=8-16) quantified by measuring LDH release, 48 hours after the beginning of the treatment with TGF-β1. * indicates significant difference from control; # indicates significant difference from 100nM rotenone alone, one-way ANOVA ($p < 0.05$).

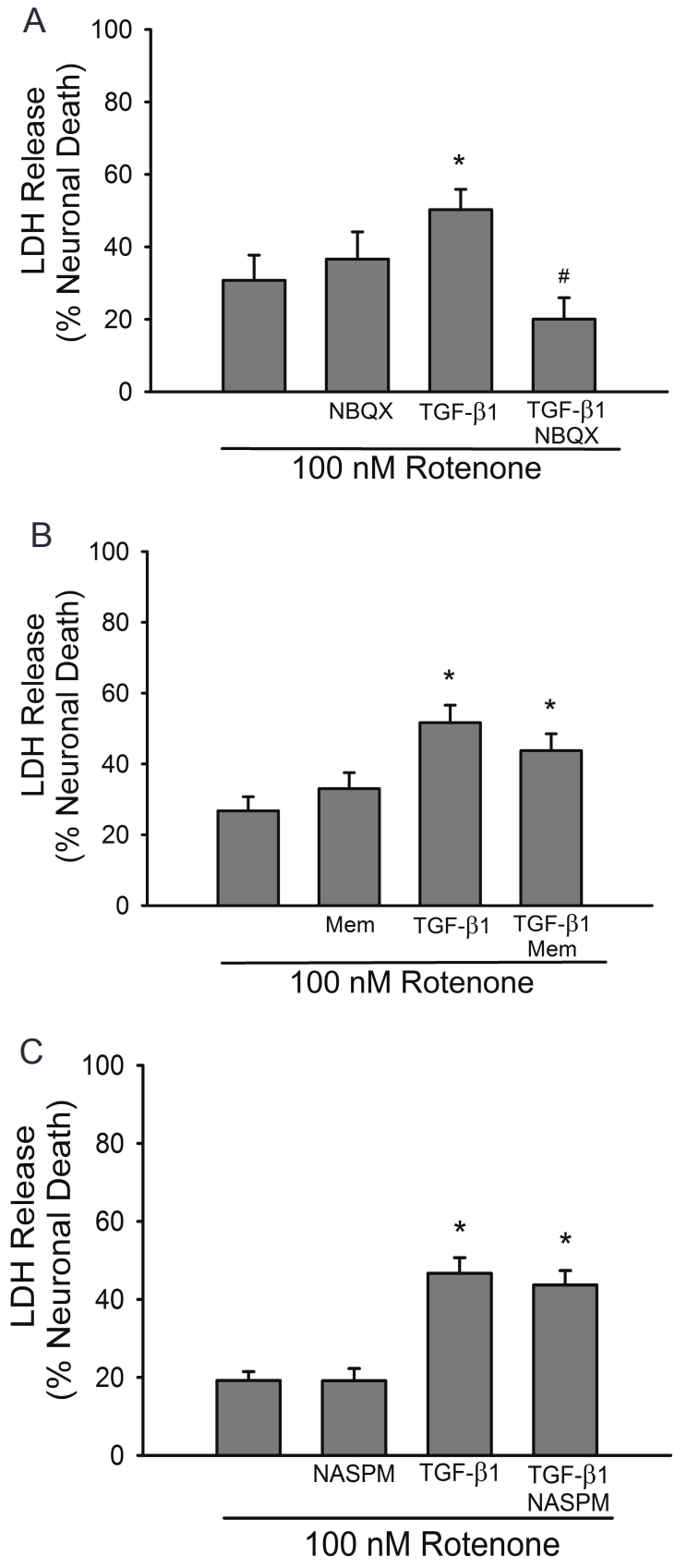

Figure 4.8 AMPA receptor antagonist, NBQX, blocks TGF-β1 enhanced rotenone-toxicity; NMDA receptor antagonist, MEM, and Ca^{2+}-permeable AMPA receptor antagonist, NASPM, do not. TGF-β1 (100ng/mL) was applied for 48 hours to mixed cortical cultures. 24 hours after TGF-β1 application, rotenone (100nM) and A) NBQX (7.5μM), B) MEM (10μM), or C) NASPM (10μM) were applied. Bars show % neuronal death (mean ± s.e.m, n=8-16) quantified by measuring LDH release, 48 hours after the beginning of the treatment with TGF-β1. * indicates significant difference from control; # indicates significant difference from 100nM rotenone alone, one-way ANOVA ($p < 0.05$).

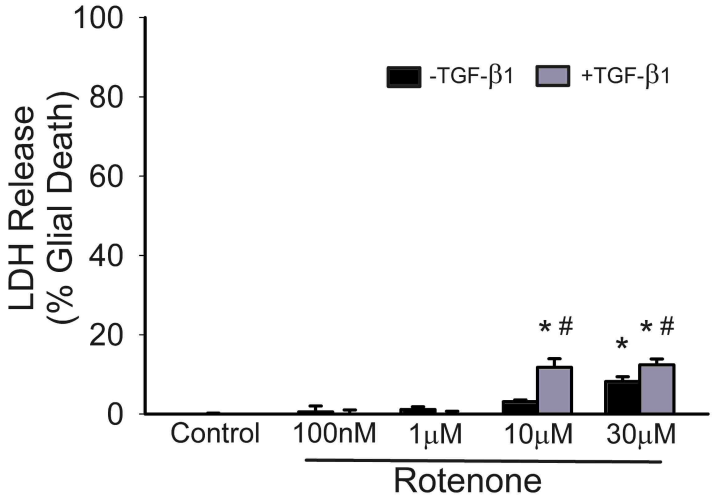

Figure 4.9 TGF-β1 enhances rotenone-induced toxicity in astrocytes. TGF-β1 (100ng/mL) was applied for 48 hours to astrocyte-enriched cultures. 24 hours after TGF-β1 application, the indicated amount of rotenone was applied. Bars show % death (mean ± s.e.m, n=8-16) quantified by measuring LDH release, 48 hours after the beginning of the treatment with TGF-β1. * indicates significant difference from control; # indicates significant difference from TGF-β1 alone, one-way ANOVA ($p < 0.05$).

Discussion

TGF-β1 is known to have many diverse effects on the central nervous system (Flanders et al., 1998) and this current study adds a novel effect of TGF-β1 to the literature. Our results show that 24-hour application of TGF-β1 increased cystine uptake through system x_c^- on astrocytes. The timecourse of system x_c^- upregulation by TGF-β1 suggests that new protein is being made; in support of this hypothesis, the protein synthesis inhibitor, cycloheximide, blocked the TGF-β1 mediated increase in system x_c^- function. Also, TGF-β1 increased xCT mRNA levels in a time dependent manner. Together these data suggest that cystine uptake is increased through system x_c^- due to increased system x_c^- expression.

TGF-β1 can signal through multiple pathways and involves two receptors, TGF-βRI and TGF-βRII. Both receptors I and II are serine/threonine kinases and TGF-βRI has multiple isoforms. TGF-β1 signaling begins when TGF-β1 binds to TGF-βRII, which recruits and phosphorylates TGF-βRI. TGF-βRI then initiates the intracellular signaling cascade (Massagué, 1998). Interestingly, in this study the TGF-β1 induced increase in system x_c^- activity requires both TGF-βRI isoforms, ALK1 and ALK5. Inhibiting one receptor only partially blocked the increase in cystine uptake, while blocking both receptors completely blocked the increase. Activation of both ALK1 and ALK 5 signaling has been observed previously, and certain biological responses have been shown to be dependent upon recruitment of both isoforms (Goumans et al., 2003; König et al., 2005).

Following TGF-βRI activation, TGF-βRI phosphorylates and activates signaling mother against decapentaplegic (SMAD) proteins. SMAD proteins are transcription factors and are the main signal transducers of TGF-β receptor activation (Massagué, 2000). More recently, however, other signaling pathways of TGF-βRI/II activation are beginning to emerge (Massagué, 2000; Vivien and Ali, 2006). Along with being serine/threonine kinases, recently it was discovered that TGF-βRI/II can also phosphorylate tyrosine, which plays a role in activating the MAPK/ERK signaling pathway (Lee et al., 2007; Zhang, 2009). In this study when added one hour prior to TGF-β1, U0126 blocked the increase in system x_c^- activity, showing that the MAPK/ERK signaling pathway was necessary for the increase system x_c^- activity. We did not test inhibitors of other known TGF-β1 signaling pathways and therefore cannot

rule out the involvement of these other pathways in TGF-β1's affects on system xc- activity.

Given the function of system x_c-, one would hypothesize that increased system x_c- activity would also lead to increased intracellular GSH levels. However, our data indicate the situation is complicated. Increased system x_c- activity on astrocytes actually led to decreased GSH levels in astrocytes. This appears to be due to TGF-β1 treatment not only increasing cystine uptake into astrocytes, but also stimulating astrocytes to release more GSH. When TGF-β1 was applied to pure neuronal cultures there was a decrease in both intracellular and extracellular GSH production. However, when astrocytes and neuronal cells were cultured together using an insert system and GSH was measured in each cell population separately, TGF-β1 application still led to a decrease in GSH levels in astrocytes, but GSH levels in the neurons were slightly higher than control levels. This finding suggests that TGF-β1 leads to the export of GSH from astrocytes so that it can be utilized by neurons. This is consistent with the well described function of astrocytes as a source of substrate for neuronal GSH production (Dringen and Hirrlinger, 2003).

This release of GSH is likely a benefit to neurons. Decreased cellular glutathione has been shown to occur in schizophrenia (Yao et al., 2006), depression (Godlewska et al., 2015), and neurodegenerative diseases (Schulz et al., 2000). In these cases it would be expected that upregulation of system x_c- would be beneficial. For example, the protective effects of TGF-β1 against oxidative stress mediated insults, such as amyloid-β toxicity (Ren and Flanders, 1996; Shen et al., 2014) could be mediated by the increased production of GSH. However, with increased system x_c- activity the potential for

increased excitotoxicity also exists. In our mixed cortical cultures excitotoxicity masked any of TGF-β1's potentially protective effects against an oxidative insult. 48-hour application of TGF-β1 alone was not toxic to neurons. However, when cultures were treated with TGF-β1 for 24 hours and then rotenone was co-applied for 24 hours, which interferes with mitochondria and increases reactive oxygen species (ROS), TGF-β1 exacerbated the rotenone-induced toxicity. We were able to block this toxicity with the general AMPA receptor antagonist NBQX, but not the calcium-permeable AMPA receptor antagonist NASPM or the NMDA receptor antagonist memantine.

These results indicate a specific enhancement of AMPA receptor mediated excitotoxicity. This finding is consistent with previous studies. TGF-β1 has been shown to be protective against NMDA receptor mediated excitotoxicity, while it has been shown to enhance the toxicity of AMPA/kainate receptor mediated excitotoxicity (Prehn and Miller, 1996). TGF-β1 protection against NMDA receptor mediated toxicity involves upregulation type 1 plasminogen activator inhibitor (PAI-1) (Buisson et al., 1998; Docagne et al., 1999). The protection by PAI-1 is due to its inhibition of tPA, which increases NMDA toxicity by cleaving the NR1 subunit leading to increased NMDA induced calcium influx (Nicole et al., 2001). The injury promoting effect could be due to TGF-β1 upregulating AMPA receptor subunits. Interestingly, upregulation of GluA2, but not the GluA4 subunits has been observed (Bae et al., 2011). Since lack of a GluA2 subunit allows calcium permeability (Hollmann et al., 1991), the upregulation of the GluA2 subunit is consistent with the results that the toxicity of TGF-β1 treatment in combination with rotenone is mediated by general AMPA receptors and not calcium permeable AMPA receptors. Another possibility is that TGF- β1 effects glutamates

affinity for AMPA receptors. Altering subunit composition or phosphorylation of the AMPA receptor could change affinity for the receptor and changes in affinity could play a role in the AMPA receptor mediated death.

The potentiation of toxicity could also be caused by changes in excitatory amino acid transporter (EAAT) function. EAATs are the mechanism by which glutamate is removed from the extracellular space and decreased EAAT function can lead to excitotoxicity (O'Shea, 2002). However, there were no changes in EAAT function, as measured by radiolabeled D-aspartate uptake (data not shown). This finding is consistent with other studies that show TGF-β1 does not effect EAAT function (Piani et al., 1993; Brown, 1999).

In the current study TGF-β1 effects on astrocytes not only led to enhanced rotenone toxicity in neurons, but it also enhanced rotenone toxicity in astrocytes. The current study provides interesting differences and similarities to that of He et al. (2015). In that paper the authors found that treating cultured cortical astrocytes with IL-1β caused an upregulation of system x_c-. They also observed increased release of GSH, but in contrast to the current study they found an increase in intracellular GSH. What is unusual in the current study is that TGF-β1 stimulates the release of GSH in astrocytes to such an extent that GSH levels in astrocytes actually decrease below control levels and can lead to enhanced rotenone toxicity. This suggests a function of astrocytes in which their purpose of providing GSH to neurons takes precedence over maintaining their own GSH levels.

The most important implication of the current study is the possibility that some of the known actions of TGF-β1 are mediated by its regulation of system x_c-. In a similar study, treatment with TGF-β1 was shown to induce death of immature cerebellar neurons

co-cultured with cerebral astrocytes (Brown, 1999). The mechanism of the toxicity appeared to be increased release of glutamate from the astrocytes as there was increased glutamate in the media. Astrocyte conditioned media also was toxic to the neurons and that toxicity was blocked by an NMDA receptor antagonist. In retrospect, the increased glutamate release was likely due to upregulation of system x_c-. We do not observe toxicity in our mixed cortical cultures with TGF-β1 treatment without the added insult of rotenone, possibly due to a high rate of glutamate uptake in our culture system.

TGF-β1 is increased in both the SOD1-G93A mouse model of ALS and in human ALS patients, and TGF-β1 levels are positively correlated with the duration of the disease (Houi et al., 2002; Iłzecka et al., 2002; Endo et al., 2015). More specifically, overproduction of TGF-β1 in astrocytes has been found to accelerate disease progression in a non-cell autonomous way and inhibiting TGF-β1 signaling can extend survival time of SOD1-G93A mice (Endo et al., 2015). Interestingly, system x_c- is increased in multiple ALS mouse models, including the SOD1-G93A model (Albano et al., 2013; Mesci et al., 2015). Similar to inhibiting TGF-β1, deleting xCT in the SOD1 mutant mouse slows disease progression (Mesci et al., 2015). It is possible that increased TGF-β1 production is responsible for increased system x_c- activity in ALS and both may be potential therapeutic targets.

System x_c- is known to be upregulated in gliomas with the consequence of making the gliomas resistant to oxidative stress (Singer et al., 2015) and the added problem of increased release of glutamate causing neuronal death surrounding the glioma leading to increased growth (Ye and Sontheimer, 1999). Because of these actions inhibitors of system x_c- are being proposed as treatment for gliomas (Chung et al., 2005;

Lo et al., 2008). TGF-β1 is also known to play an important role in promoting glioma growth and antagonists of TGF-β1 signaling are being tested in clinical trials for the treatment of gliomas (Joseph et al., 2013; Kaminska et al., 2013). Given our results it is interesting to speculate that some part of TGF-β1's stimulation of glioma growth may be due to its upregulation of system x_c-. This undoubtedly would not be the only mechanism of TGF-β1 action as it has also been shown to promote tumor development by contributing to angiogenesis (Ueki et al., 1992).

Another possibility is that in certain disease states there is compromised TGF-β1 signaling leading to decreased system x_c- function, decreased cellular GSH and therefore disrupted function. This may be the case in Huntington's disease. TGF-β1 is decreased in post-mortem brain tissue from Huntington's disease patients, in the R6/2 HD mutant mouse model of Huntington's disease, and in cultured astrocytes expressing the mutated form of the huntington gene (Battaglia et al., 2011). Interestingly, xCT levels are also decreased in the R6/2 HD mouse and cells with the huntington gene mutation have increased oxidative stress in response to system x_c- inhibition (Frederick et al., 2014). These results raise the possibility that the oxidative stress observed during Huntington's disease (Li et al., 2010) may be mediated by decreased system x_c- activity as a result of decreased levels of TGF-β1.

In astrocyte cultures TGF-β1 increases system x_c- activity through the MAPK/ERK pathway. It also decreases GSH levels in astrocytes by increasing GSH export, even to the detriment of the astrocytes. The exported GSH may be utilized by neurons; however, TGF-β1 enhances rotenone-induced excitotoxicity in neurons, through AMPA receptors. Dysregulation of TGF-β1 and system x_c- occur in many of the same

diseased states and it is possible that some of the known actions of TGF-β1 may be mediated by its regulation of system x_c^-.

CHAPTER V

DISCUSSION

General Discussion

The dual actions of system x_c^- make it of great interest in many neurodegenerative diseases where oxidative stress and excitotoxicity are involved. The studies described in this thesis were the first to observe that system x_c^- is dysregulated in an ALS model and that intracellular cysteine levels may play an important role in regulating system x_c^- function. We also show that TGF-β1 upregulates system x_c^-, but that this many have negative consequences on cell death under conditions of high oxidative stress. The following section discusses some of the major findings of this thesis and the broader implications of how regulating system x_c^- affects neuronal death. It also discusses the limitations of the work done, as well as future studies that are needed in order to further our understanding of system x_c^-.

THE REGULATION OF SYSTEM X_C^-

The Age Factor

While studying system x_c^- function in SOD1-G93A mutant mice, we observed that its activity decreased with age in non-transgenic littermates (Chapter IV). The role system x_c^- plays in development and aging is largely unknown. Glutathione (GSH) levels are high in neuronal and non-neuronal cells in the immature brain, as well as in developing neurons of the mature brain (Lowndes et al., 1994; Shih et al., 2006; Sun et al., 2006). GSH is important for proliferation, differentiation, and viability of neurons

during development since these are times where metabolic and oxidative demand is high (Dalton et al., 2004). System x_c^-'s import of cystine is directly tied to GSH synthesis (Sagara et al., 1993a); therefore system x_c^- may play an important role in supplying developing neurons with the high levels of GSH they need. In support of this idea, studies show that neuronal death induced by glutamate in immature neurons is a consequence of increased oxidative damage caused by a depletion of GSH resulting from system x_c^- inhibition (Murphy et al., 1990; Shih et al., 2003; Shih et al., 2006). Astrocytes, fibroblasts, and meningeal cells cultured from both xCT knockout ($xCT^{-/-}$) mice and mice with the subtle gray pigmentation mutant phenotype (sut/sut), a xCT loss of function mutation, are unable to proliferate *in vitro* and need the reducing agent β-mercaptoethanol in order to grow (Sato et al. 2005; Shih et al. 2006). Similarly, our mixed cortical cultures do not survive if we apply sulfasalazine (SSZ), a system x_c^- inhibitor (Gout et al., 2001), on days *in vitro* 1-3 (data not shown).

While these data support the hypothesis that system x_c^- activity is important during development, western blot analysis suggests that expression of xCT, the functional subunit of system x_c^-, is much lower in the developing brain compared to the adult brain (Shih et al., 2006; La Bella et al., 2007). Also, differing effects of xCT knockout and xCT mutation on brain morphology have been reported; Shih et al. (2006) report that sut/sut mice show brain atrophy. However, normal brain appearance has been reported in $xCT^{-/-}$ and sut/sut mice, suggesting that xCT is not critical for development *in vivo* (Sato et al., 2005; Massie et al., 2015). In the adult brain, a study by Liu et al. (2007) used sut/sut mice to assess cellular proliferation in the subventricular zone and dentate gyrus, two regions where neuronal development continues into adulthood. The sut/sut mice

showed a small, non-significant, decrease in cell proliferation of the subventricular zone, but increased cell proliferation in the dentate gyrus. They concluded that xCT is not essential to cell proliferation in the adult brain. Overall, it appears that system x_c^- is critical for the proliferation and survival of cells *in vitro*, but lack of system x_c^- may be able to be compensated for *in vivo*.

System x_c^- might also play a role in aging. The "free radical theory of aging" is one of the leading theories on the mechanism behind aging. The basic idea is that aging occurs as a result of irreversible damage caused by an increase in reactive oxygen species (ROS) (Kregel and Zhang, 2007). ROS increase with age in many organ systems, including the brain (Driver et al., 2000; Kregel and Zhang, 2007). Along with an increase in ROS, GSH levels decrease in many different cell types, including hippocampal neurons, during aging (Lieshout and Peters, 1998; Erden-Inal et al., 2002; Parihar et al., 2008). In fact, overall GSH levels in the adult brain are significantly lower compared to the immature brain (Lowndes et al., 1994; Shih et al., 2006). System x_c^- plays a key role in GSH synthesis, therefore it is possible that it plays a role in the aging process. One hypothesis would be that the decrease in GSH levels seen during aging is due to a decrease in system x_c^- activity. In support of this hypothesis we observed a significant decrease in system x_c^- activity in the spinal cord slices of SOD1-G93A non-transgenic littermates between the ages of 55 days and 70 days; this lower level of activity was maintained through 130 days (Albano et al., 2013). Work in system x_c^- knockout mice may also support this hypothesis. 8-10 week old $xCT^{-/-}$ mice have a shift in their cystine/cysteine redox balance (Sato et al., 2005). They have higher levels of cystine in their plasma, but no change in cysteine plasma levels. This shift in redox

balance is similar to what is seen in elderly patients (Jones et al., 2002), which lead Sato et al. (2005) to speculate that aging may be accelerated in xCT$^{-/-}$ mice.

While the role of system x_c^- during development and aging is still unknown, its role in maintaining balance between ROS and GSH is well known and makes it likely to be important in both of these life stages. A more detailed study of different age ranges (embryonic, young pup, adolescent, middle-aged adult, aged) needs to be performed in order to have a better understanding of how system x_c^- changes with age.

Cystine/Cysteine Redox Balance

In chapter three of this thesis we provide evidence that suggests cysteine is a more important regulator of system x_c^- function than GSH levels. System x_c^-'s functions of providing the cell with cystine for GSH synthesis and regulating extracellular glutamate, often masks its function of regulating the extracellular cystine/cysteine redox state that surrounds the cellular membrane. Cystine taken up by system x_c^- is reduced to cysteine. Cysteine can then be used to synthesize GSH, which can leave the cell and contribute to the reduced extracellular environment (Wang and Cynader, 2000; Dringen and Hirrlinger, 2003). However, some of the cysteine is also directly exported from the cell via system ASC, which plays an important role in maintaining the extracellular redox environment independent of the GSH (Bannai and Ishii, 1982; Jones et al., 2002; Anderson et al., 2007). Given how important cysteine is to regulating the redox environment of the cell and how system x_c^- helps to maintain the cystine/cysteine balance, it is easy to see why cysteine would be an important regulator of system x_c^- function.

A study by Banjac et al. (2008) lends support to our conclusions. They found that expressing xCT in lymphoma cells, which normally take up little cystine, was able to

protect these cells from oxidative stress. Interestingly however, expressing xCT did not increase GSH levels, rather it increased both intracellular and extracellular cysteine levels. They concluded that the protection by xCT was not due to GSH as predicted; rather it was due to increased cysteine levels. Similarly, glutamate-cysteine ligase (GCL) knockout cells, which are unable to synthesize GSH and therefore die within 48 hours, are able to proliferate if xCT is overexpressed due to high intracellular and extracellular cysteine levels (Mandal et al. 2010).

The mechanism by which cysteine is downregulating system x_c^- still remains to be determined. One possibility is that it is inhibiting the transcription factor nuclear factor erythroid-derived 2 (Nrf-2). Nrf-2 is the main regulator of system x_c^- and increases transcription of xCT in response to oxidative stress (Ishii et al., 2000; Sasaki et al., 2002; Lewerenz et al., 2009). Another possibility is that cysteine inhibits the phosphorylation of xCT by protein kinase A (PKA). Inhibiting PKA has been shown to decrease system x_c^- activity in *ex vivo* striatal punches (Baker et al. 2002). Future studies need to be performed in order to determine the mechanism behind cysteine regulation of system x_c^-.

Neuronal Regulation

In our culture system we observed that astrocytes co-cultured with neurons that are killed (mixed cultures treated with 500μM NMDA) have a higher level of ^{14}C-cystine uptake than astrocytes cultured without neurons (astrocyte-enriched glial cultures). This observation suggests that when cultured together, neurons release a signal that contributes to the regulation of system x_c^- on astrocytes. To determine if this hypothesis was correct we compared the function of system x_c^- in astrocyte-enriched glial cultures that were

treated with media from pure neuronal cultures (PNC) to those treated with media from other astrocyte-enriched glial cultures. Astrocytes treated with PNC conditioned media for at least 24 hours had significantly higher ^{14}C-cystine uptake than those treated with glial media (Figure 5.1A). The system x_c^- inhibitors SSZ and (S)-4-carboxyphenylglycine (CPG) blocked the increase in ^{14}C-cystine uptake showing that it was through system x_c^- (Figure 5.1B). To determine what the signal from neurons was that led to the increase in system x_c^- function, we tested receptor antagonists against factors that we know upregulate system x_c^- on astrocytes in our culture system; specifically, we tested antagonists against TGF-β1, FGF-2, and PACAP. However, we were unable to block the increase in ^{14}C-cystine uptake with any of the antagonists tested (data not shown). While we were unable to determine the exact neuronal signal, we were able to determine that the neuronal signal mediates the increase in system x_c^- function through the MEK/ERK pathway (Figure 5.1C).

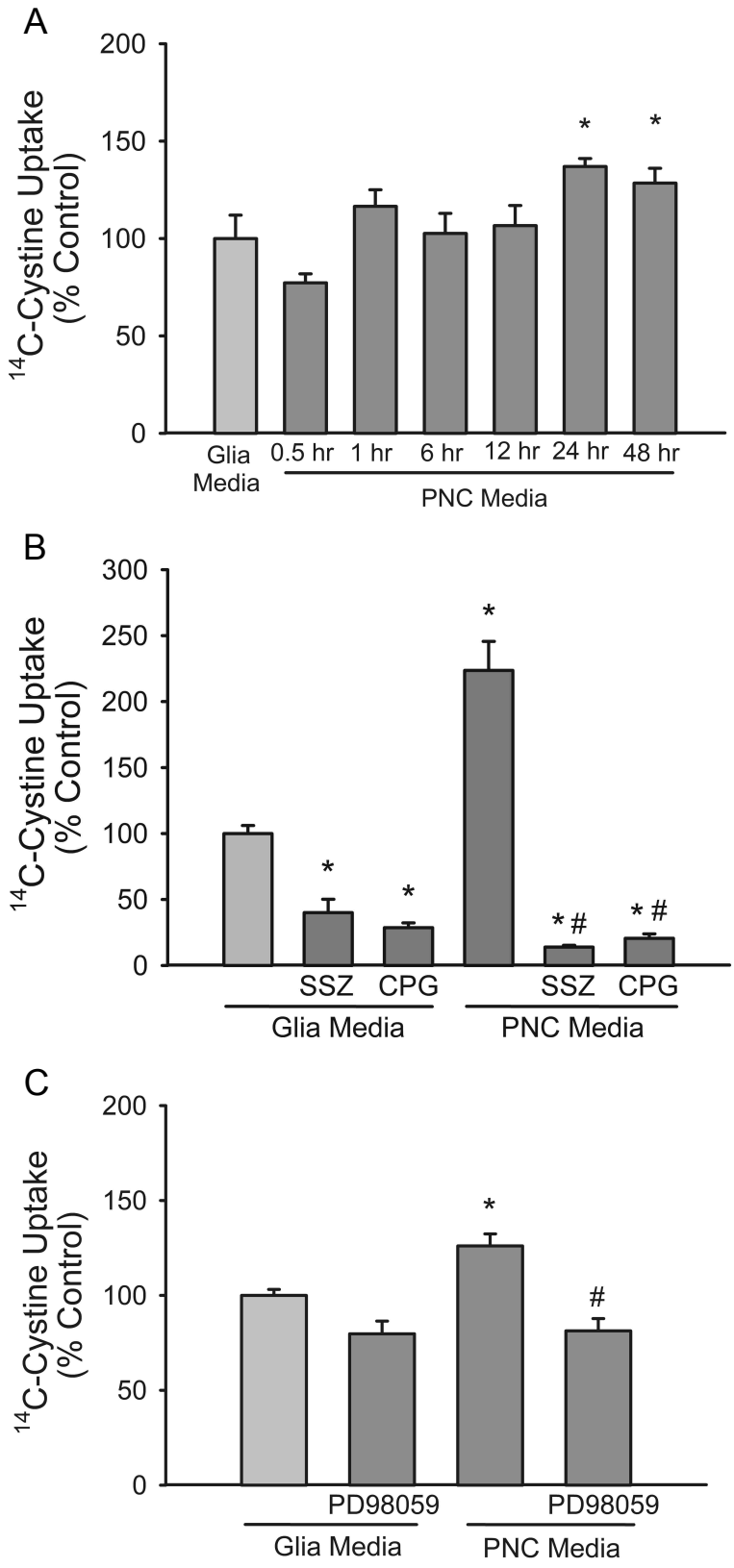

Figure 5.1 A) Astrocyte-enriched glial cultures treated with PNC media for 24 or 48 hours had significantly higher levels of ^{14}C-cystine uptake than those treated with glial media. B) The system x_c- inhibitors SSZ and CPG blocked the PNC media mediated increase in ^{14}C-cystine uptake. C) The increase in ^{14}C-cystine uptake mediated by PNC media is blocked with the MEK/ERK pathway antagonist, PD98059. Results are expressed as mean±s.e.m. (n=8-16) after normalizing to untreated glial media uptake. * indicates significantly different from glial media; # indicates significant difference from PNC media, one-way ANOVA ($p < 0.05$).

Recently, a study by Kong et al. (2016) found that inhibiting the PACAP receptor with $PACAP_{6-38}$ blocked neuron-induced upregulation of system x_c-. Although this finding is in contrast to our observations there are some marked differences between the two studies. First, they are using cortical cultures from rats, while we use mice. Second, the culturing methods between the two studies are quite different. Finally, while we also used the PACAP6-38 antagonist to try to block the neuronal signal, the Kong et al. (2016) study used a concentration of PACAP6-38 that can block multiple receptors; we applied a much lower concentration.

Even though we were unable to determine the exact signaling molecule by which neurons regulate system x_c- function on astrocytes, the observation that there is neuronal regulation is important. As previously discussed, the neuron-astrocyte interaction plays a large role in synaptic transmission (Araque et al.,1999; Newman, 2003; Bridges et al., 2012a). For example, the glutamate released from system x_c- into the extrasynaptic space is able to regulate the amount of glutamate released in the synapse, through mGluR2/3 activation (Baker et al., 2002). Glutamate released by system x_c- can also regulate synaptic strength by reducing the number of postsynaptic AMPA receptors (Williams and Featherstone, 2014). The ability for neurons to modulate the amount of glutamate released extrasynaptically through system x_c- may be a way in which they are able to control synaptic strength and transmission.

Not only are neuron-astrocyte interactions important for regulating synaptic transmission, but astrocytes also provide antioxidant support to neurons by supplying them with GSH precursors (Sagara et al., 1993a; Dringen et al., 1999; Wang and Cynader, 2000; Shih et al., 2003). The antioxidant support provided by

astrocytes begins with the transport of cystine into astrocytes through system x_c^- (Kranich et al., 1998). Therefore, it is essential that neurons are able to signal astrocytes to increase system x_c^- function when they are in need of increased antioxidant support. Determining the specific signal released by neurons is of importance because not only will it provide insight into specific situations when neuronal regulation of system x_c^- is necessary, but it may also provide a therapeutic target by which system x_c^- function can be up- or downregulated.

TGF-β1

TGF-β1 signaling is dysregulated in response to injury and several neurodegenerative diseases (Pratt and McPherson, 1997; Flanders et al., 1998; Vivien and Ali, 2006). While it is mainly thought to be neuroprotective, some evidence suggests that it might potentiate neuronal injury (Prehn and Miller, 1996; Endo et al., 2015). In chapter four of this thesis, we presented data showing that TGF-β1 increases system x_c^- function on astrocytes. This effect requires the activation of two TGF-βR1 isoforms, ALK5 and ALK1, which in turn activate the MAPK/ERK pathway. This novel action of TGF-β1 may provide further insight into its role in neuronal protection/death.

TGF-β1, GSH, and Astrocyte Toxicity

The import of cystine through system x_c^- activity is directly tied to GSH synthesis (Sagara et al., 1993a). GSH is able to protect astrocytes from various insults (Kim et al., 2003; Liddell et al., 2006). However, when we compare the effects of TGF-β1 to the cytokine interleukin-1β (IL-1β) there are some marked differences in the way that GSH is regulated and its ability to protect astrocytes. While both TGF-β1 and IL-1β increase

system x_c- function on astrocytes (Fogal et al., 2007; Jackman et al., 2010; Chapter IV), 24-hour application of TGF-β1 decreases intracellular GSH levels in astrocytes, while IL-1β increases intracellular GSH levels (He et al., 2015). He et al. also shows that the IL-1β-induced increase in GSH production protects astrocytes from ROS-inducing $FeSO_4$ and tert-butyl hydroperoxide (tBOOH) exposure. Not only is TGF-β1 unable to protect astrocytes from rotenone-induced toxicity, it actually exacerbates its toxicity.

Cytokines are known to have many diverse effects. One possibility for the marked differences in TGF-β1 and IL-1β's effects on astrocyte protection could be due to differential effects on GSH export. While both TGF-β1 and IL-1β increase GSH export into the extracellular space (He et al., 2015; Chapter IV) they could regulate the GSH exporter, multidrug resistant protein 1 (MRP-1), differently. IL-1β application does not change the expression (Ronaldson et al., 2010) or function (He et al., 2015) of MRP-1 in astrocytes. The effects of TGF-β1 on MRP-1 expression are unknown, however, the MRP-1 inhibitor MK-571 brings intracellular GSH levels back up to control levels (Figure 5.2), suggesting TGF-β1 does not affect MRP-1 function. Although MK-571 was not able to increase GSH levels when co-applied with TGF-β1 to the same level as MK-571 under control conditions, we used a fairly low concentration of MK-571 (10μM).

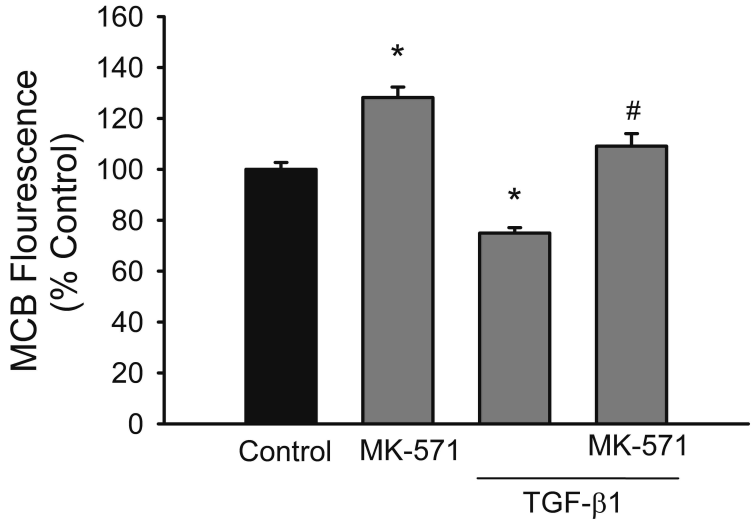

Figure 5.2 The multidrug resistant protein 1 (MRP-1) inhibitor, MK-571 (10μM), blocked the decrease in intracellular GSH levels caused by TGF-β1. Results are expressed as mean±s.e.m. (n=8-16) after normalizing to control. * indicates significant difference from control, # indicates significant difference from TGF-β1, one-way ANOVA ($p < 0.05$).

Oxidative stress could be another reason for the differential effects of TGF-β1 and IL-1β on astrocyte protection. He et al. (2015) has shown that the GSH:GSSG ratio increases with time in the presence of IL-1β. They use this ratio to show that IL-1β is not causing an increase in oxidative stress in their astrocytes. An increase in oxidative stress caused by TGF-β1 application could lead to the decrease in intracellular GSH levels and account for the increase in rotenone-induced toxicity. TGF-β1's effects on oxidative stress are explored in the next section.

TGF-β1 and Oxidative Stress

Studies show that TGF-β1 protects hippocampal neurons against oxidative insult (Prehn et al., 1994; Henrich-Noack et al., 1996). However, TGF-β1 was found to increase mitochondrial ROS production in several cell types, leading to increased oxidative stress (Liu and Desai, 2015). It is also well established that oxidative stress

can increase system x_c- activity (Kim et al., 2001; Dun et al., 2006; Mysona et al., 2009). In order to determine if the increase in system x_c- activity induced by TGF-β1 was mediated by oxidative stress in astrocyte-enriched glial cultures we applied TGF-β1 (100ng/mL) and measured ROS levels using the DCF assay. Initially we saw an early increase (at hours 1 and 3) in ROS production after TGF-β1 application (data not shown). We were able to block the increase in ROS levels at 1 hour with the MAPK/ERK antagonist, U0126 (data not shown).

These data suggest that TGF-β1 activates the MAPK/ERK pathway, which then leads to an increase in ROS production. Since the increase in ROS production was seen after a 1-hour application of TGF-β1 but back down to control levels by 24 hours and the increase in ^{14}C-cystine uptake through system x_c- occurred 24 hours after application of TGF-β1, we hypothesized that the increase in oxidative stress was mediating the increase in system x_c-. However, we were unable to replicate the increase in ROS production with 1-hour application of TGF-β1 (data not shown). Also, in the ^{14}C-cystine uptake assay we were unable to block the TGF-β1 mediated increase in system x_c- activity with free radical scavengers. We tested other free radical scavengers, but were still unable to block the increase in system x_c- activity. Therefore, while TGF-β1 inducing an increase in oxidative stress, which leads to the upregulation of system x_c- is an attractive hypothesis, we were not able to confirm that hypothesis. Limitations of the DCF assay to measure all types of oxidative stress (Karlsson et al., 2010) and the inability of free radical scavengers to eliminate all ROS means that the hypothesis may be correct, but we were unable to confirm it.

THE EFFECT OF SYSTEM X_C- FUNCTION ON NEURONAL DEATH

TGF-β1's Effects on Neuronal Death

Physiologically TGF-β1's expression is very low under basal conditions; its expression and release are upregulated in many neurodegenerative diseases and in response to CNS injury (Pratt and McPherson, 1997; Flanders et al., 1998; Vivien and Ali, 2006). Therefore, we were interested in TGF-β1's effects on neuronal death in concert with an increase in oxidative stress, a hallmark of many neurodegenerative diseases. Even though TGF-β1 caused astrocytes to export GSH, which should provide neuroprotection against the oxidative stress induced by rotenone, TGF-β1 exacerbated rotenone-induced neuronal death. The AMPA receptor antagonist, NBQX (7.5μM), but not the NMDA receptor antagonist, memantine (10μM), was able to block the toxicity caused by the addition of TGF-β1. These results suggest that TGF-β1 exacerbates rotenone-induced toxicity through an AMPA receptor-mediated excitotoxic mechanism.

In order to determine if the excitotoxicity was caused by an increase in glutamate release due to TGF-β1's increase in system x_c- activity, blocking system x_c- with an inhibitor would be the next logical experiment. However, since rotenone toxicity is caused by increased oxidative stress (Li et al., 2003; Sherer et al., 2003) and blocking system x_c- activity with an inhibitor would block GSH production, we found that the system x_c- inhibitor SSZ greatly potentiated normal rotenone toxicity (data not shown). Therefore, due to the complexity of the experiment, we were unable to directly show that the increased excitotoxicity seen with TGF-β1 treatment during rotenone exposure was due to an increase in glutamate released through system x_c-. Another way to approach

answering this question would be to measure extracellular glutamate levels when TGF-β1 is applied, using HPLC. However, in our mixed cultures, which have high glutamate uptake, we have never been able to measure system x_c^- mediated glutamate release.

Although we could not obtain direct evidence that the TGF-β1-induced increase in system x_c^- activity on astrocytes was responsible for the enhanced rotenone-induced neuronal death, a study by Brown (1999) lends support to this hypothesis. Brown found that TGF-β1 causes excitotoxicity in cerebellar neurons only if astrocytes are present. He concluded that TGF-β1 acted upon astrocytes to cause neuronal death through an increase in extracellular glutamate. It is possible that the increase in glutamate was due to an increase in system x_c^-.

TGF-β1 may also exert its effects through mechanisms other than system x_c^- that contribute to the excitotoxicity that Brown (1999) saw in cerebellar neurons and that we see with rotenone-induced toxicity in cortical neurons. One such possibility is that TGF-β1 causes excitotoxicity by decreasing the astrocytes' ability to clear glutamate. However, along with others, we found that TGF-β1 did not affect astrocytes' ability to clear extracellular glutamate through excitatory amino acid transporters (EAATs) (Piani et al., 1993; Brown, 1999).

Another possibility is that TGF-β1 could be affecting AMPA receptor expression and/or function. TGF-β1 increases GluA2-containing AMPA receptor expression, but not GluA4-containing AMPA receptors in the hippocampus (Bae et al., 2011), which is consistent with our rotenone-enhanced excitotoxicity working through general AMPA receptors, but not Ca^{2+}-permeable AMPA receptors. The question still remains, however, why is TGF-β1 enhanced excitotoxicity AMPA-mediated and not NMDA-mediated?

Other studies show a similar mechanism for TGF-β1 mediated toxicity; TGF-β1 protects against NMDA toxicity but enhances AMPA toxicity (Prehn et al., 1993; Prehn and Miller, 1996; Buisson et al., 1998). The mechanism of TGF-β1 protection against NMDA toxicity has been partially determined (Prehn and Krieglstein, 1994; Buisson et al., 1998), while the mechanism of TGF-β1exacerabtion of AMPA toxicity remains unclear.

The Effect of Increasing System x_c^- Function on Neuronal Death

The dual actions of system x_c^- and its ability to be regulated by a wide range of compounds and pathologies suggest that it can play an important role in neuronal protection and death. System x_c^- takes up cystine from the extracellular space and uses it in the formation of GSH, which can protect cells from oxidative stress. In fact, immature primary cortical neurons (Murphy et al., 1990), fetal brain cells (Sagara et al., 1993b), and neuronal cell lines (Miyamoto et al., 1989; Murphy et al., 1989), rely heavily on system x_c^- to protect them from oxidative glutamate toxicity. Oxidative glutamate toxicity occurs when high levels of extracellular glutamate are applied to cells that lack glutamate receptors; instead of causing excitotoxicity, high levels of glutamate competitively inhibit the uptake of cystine through system x_c^-, causing GSH depletion, resulting in oxidative stress and cell death (Murphy et al., 1989; Ratan et al., 1994). Overexpressing xCT or Nrf-2 in astrocytes protects immature neurons from oxidative glutamate toxicity (Shih et al., 2003; Shih et al., 2006). Ceftriaxone, an antibiotic that upregulates system x_c^- activity through the Nrf-2 pathway, has also been shown to protect against oxidative glutamate toxicity in HT22 cells, which are not sensitive to excitotoxicity (Lewerenz et al., 2009).

System x_c- can also protect against other types of oxidative stress. Nrf-2 overexpression *in vitro* protects immature neurons from hydrogen peroxide (H_2O_2) by increasing many genes involved in GSH sythesis, including xCT (Shih et al., 2003). The growth factors insulin-like growth factor (IGF-1) and TGF-β1 increase system x_c- activity on dental pulp cells, which leads to protection from oxidative stress induced by different dental materials (Pauly et al., 2011). When exposed to diethyl maleate (DEM), which depletes cellular GSH, glioma cells increase xCT expression and system x_c- activity (Kim et al., 2001). System x_c- activity is needed in order to maintain the high levels of GSH necessary for glioma cells to grow (Chung et al., 2005; Chung and Sontheimer, 2009).

While the above examples show increased system x_c- function is protective against different types of oxidative stress, it is important to point out that immature neurons, dental pulp cells, and glioma cells, are not sensitive to excitotoxicity. Mature neurons, however, are susceptible to glutamate-induced excitotoxicity and an increase in extracellular glutamate released through system x_c- can cause excitotoxicity. The growth factors TGF-β1 and fibroblast growth factor-2 (FGF-2) increase system x_c- activity on astrocytes (Liu et al., 2012; Liu et al. 2014; Chapter IV). TGF-β1 enhances rotenone-induced neuronal toxicity through AMPA receptors. Similarly, long-term application of FGF-2 leads to AMPA receptor mediated neuronal death. IL-1β also upregulates system x_c- on astrocytes, which potentiates neuronal death induced by hypoxia and glucose deprivation (Fogal et al., 2007; Jackman et al., 2010; Jackman et al., 2012). Upregulation of system x_c- on microglia can also cause excitotoxicity in neurons. Microglia activation by amyloid precursor protein (Barger and Basile, 2001) and aggregated amyloid beta 1-

40 (Qin et al., 2006) increase glutamate released by system x_c-, which leads to neuronal death. Lipopolysaccharide (LPS) can also activate microglia and cause oligodendrocyte toxicity through increased system x_c- (Domercq et al., 2007).

While these studies suggest that increasing system x_c- function has negative effects on neuronal death, there is no evidence that driving system x_c- alone is lethal to neurons. In fact, under basal conditions our culture media contains 100μM cystine, which is surely driving system x_c- function. Also applying TGF-β1 (Chapter IV), IL-1β (Fogal et al., 2005) or PACAP (Resch et al. 2014) alone for 24-48 hours, all of which increase system x_c- function, does not lead to neuronal death. In all of the above-mentioned studies where upregulation of system x_c- led to neuronal death, there was also another stressor involved: growth factor activation, rotenone, hypoxia, glucose deprivation, amyloid beta, or LPS. Taken together, the data suggests that while driving system x_c- alone does not cause neuronal death, increased activity on non-neuronal cells, in concert with a stressor, can lead to neuronal death.

System x_c- in Amyotrophic Lateral Sclerosis

One of the most interesting results of this thesis is that system x_c- function is significantly increased in the SOD1-G93A mutant mouse at 70 days of age (Albano et al., 2013). This was the first study to show that system x_c- activity was dysregulated in an ALS model. However, with system x_c-'s ability to regulate both GSH production and glutamate release the key question remains: does the upregulation of system x_c- at 70 days promote neuronal protection or does it contribute to the neuronal death seen in the SOD1-G93A mutant mouse? As discussed in chapter two of this thesis, elevated levels of oxidative stress have been seen in the spinal cord of SOD1-G93A mice starting at 60

days of age (Liu et al., 1998). These data, along with changes in the GSH:GSSG ratio in the spinal cord of 80 day old SOD1-G93A mice (Chi et al., 2007) suggest that the elevated system x_c^- activity we found in 70 day old SOD1-G93A mice is induced by oxidative stress in order to increase GSH levels to protect against ROS.

In line with this hypothesis that system x_c^- is upregulated in ALS in order to provide antioxidant protection, there are a few studies that have assessed the potential therapeutic effects of N-acetylcysteine (NAC) in models of ALS. NAC is a cysteine pro-drug that drives system x_c^- activity (Kupchik et al., 2012). The results from studies using NAC to delay symptom onset and increase survival of SOD1-G93A mice have been mixed. One study that administered NAC orally reported improved rotorod performance, which is indicative of delayed symptom onset, and increased survival (Andreassen et al., 2000). However, another study that administered NAC orally in one set of animals and subcutaneously in another set of animals, did not report delayed symptom onset or increased survival in either group of animals (Jaarsma et al., 1998). The difference seen between these two studies may be due to variation in SOD1-G93A copy number. The first study used SOD1-G93A mice with a fast symptom progression, which is due to a high number of SOD1-G93A copies. The later study used mice with a lower number of SOD1-G93A copies, which resulted in a slower progression of the disease.

There is one randomized, double-blind study in human ALS patients being treated with NAC. Overall, NAC provided a small, non-significant increase in survival, with no evidence of reduction in disease progression (Louwerse et al., 1995). However, when divided into subgroups, NAC increased survival of patients with limb onset of the disease and decreased survival of patients with bulbar onset. Interestingly, riluzole, a drug that

exerts antiglutamatergic actions and is the only FDA approved drug to treat ALS, also has different effects on these subgroups. Riluzole increased survival in patients with bulbar onset but not patients with limb onset (Bensimon et al., 1994; Zoccolella et al., 2007), suggesting that excitotoxicity plays a larger role in patients with bulbar onset. It is possible that increased system x_c^- function may be particularly pronounced in ALS patients with bulbar onset, in which case driving system x_c^- with NAC could lead to excitotoxicity that overwhelms the benefit of increased cystine uptake.

While system x_c^- may be upregulated in order to provide antioxidant protection, upregulation also leads to increased glutamate release and could have excitotoxic consequences. A study using SOD1-G37R mutant mice, which phenotypically have a much slower progression of the disease than SOD1-G93A mice, observed an increase in xCT expression in microglia at the onset and symptomatic stages of disease progression (Mesci et al., 2015). Using SOD1-G37R mice that lack xCT (SOD1-G37R:$xCT^{-/-}$) they found that although symptoms appeared earlier in SOD1-G37R:$xCT^{-/-}$ mice, the disease progressed more slowly. During the symptomatic stage, SOD1-G37R:$xCT^{-/-}$ mice maintained grip strength longer and lived longer then SOD1-G37R:$xCT^{+/+}$ mice; they also had more surviving motor neurons at the end stage of the disease. Mesci et al. hypothesize that SOD1-G37R:$xCT^{-/-}$ mice have a slower disease progression due to less glutamate being released from microglia, since many studies have shown that activated microglia have increased glutamate release via system x_c^- (Piani and Fontana, 1994; Barger and Basile, 2001; Mesci et al., 2015). However, they did not directly measure glutamate levels in the SOD1-G37R:$xCT^{-/-}$ mice.

With the dual nature of system x_c^- it is no surprise that it may play a very complex role in ALS. System x_c^- function is upregulated in both SOD1-G93A and SOD1-G37R mice; however, we found that in SOD1-G93A mice it is upregulated at the presymptomatic stage, while in the SOD1-G37R mice it is upregulated at the onset and symptomatic stages (Albano et al., 2013; Mesci et al., 2015). The differences in disease progression likely have a large impact on when system x_c^- is upregulated and the effects this upregulation has in the disease. To this point, as previously described, treating SOD1 mutant mice and human ALS patients with NAC yielded different results. The differences seen in all of these studies may be due to differences between the progression of the disease (in the mutant mice) and onset subgroup (in humans). Further research into the role system x_c^- plays during the different stages of disease progression may aid in understanding the complexity of system x_c^- in ALS.

Based on the available evidence, one might speculate that in SOD1 mutant mice system x_c^- may be initially upregulated in response to increased oxidative stress in order to provide antioxidant protection, but eventually it may contribute to the excitotoxicity of motor neurons through increased glutamate release. Inhibiting system x_c^- at various time points in ALS disease progression and assessing disease onset, symptoms, and survival rate would be one way to determine if increasing system x_c^- is beneficial early on, but harmful at later stages of the disease. The Food and Drug Administration (FDA) has approved the system x_c^- inhibitor SSZ for the treatment of inflammatory bowel disease. One potential problem with using SSZ is that blocking system x_c^- may lead to a decrease in GSH levels, which would mask any of its potentially protective effects. The earlier symptom onset seen in SOD1-G37R:xCT$^{-/-}$ mice support this idea (Mesci et al., 2015).

SSZ administration in combination with L-2-oxo-4-thiazolidine carboxylate (OTC) may circumvent this problem. OTC, also known as Procysteine, is an L-cysteine precursor; it increases cysteine levels in the brain by being taken up into cells and converted into L-cysteine by the enzyme 5-oxoprolinase (Anderson and Meister, 1989). Since cysteine is the precursor to GSH, studies have shown that administration of OTC is able to increase GSH levels both *in vitro* and *in vivo* (Kranich et al., 1998; Mesina et al., 1989). Administering SSZ and OTC at the right time and in the correct dosage has the potential to block the excitotoxic effects the increase in system x_c^- might be having, while supplying the cells with the antioxidant support that blocking system x_c^- might remove.

The ability of SSZ to reach the brain and spinal cord is another potential concern when considering its therapeutic potential. Oral administration of SSZ is metabolized by bacteria found in the gut, into sulfapyridine and 5-aminosalicylic acid, which do not inhibit system x_c^- (Azadkhan et al., 1982; Gout et al., 2001). However, intraperitoneal injection of SSZ has been shown to suppress primary brain tumor growth by inhibiting system x_c^- (Gout et al., 2001; Chung et al., 2005) and it has also been shown to dose-dependently reduce extracellular glutamate levels in the prefrontal cortex (Lutgen et al., 2014). Together these studies suggest that SSZ is able to cross the blood brain barrier and inhibit system x_c^- when administered intraperitoneally.

Our findings in the SOD1-G93A mouse model of ALS add to a growing literature that shows dysregulation of system x_c^- function in many neurodegenerative diseases. In most neurodegenerative diseases, with the exception of Huntington's disease, system x_c^- function is increased; evidence suggests that this increase may contribute to the neuronal death seen in many of these diseases. Although *in vivo* evidence is still needed, the

damage is likely caused by increased glutamate release into the extrasynaptic space, which leads to an overactivation of glutamate receptors and cell death. In the EAE model of multiple sclerosis pharmacological inhibition of system x_c^- decreased disease severity (Evonuk et al., 2015); and in one study of the 6-OHDA model of Parkinson's disease $xCT^{-/-}$ mice had decreased dopaminergic cell death (Massie et al., 2011). These studies point to system x_c^- as a potential therapeutic target by which to slow progression of many neurodegenerative diseases.

Increasing System x_c^- and Neuronal Death – A Summary

As previously discussed, there is no evidence that increasing system x_c^- activity alone leads to cell death. However, upregulation of system x_c^- on non-neuronal cells, in a stressed or neurodegenerative diseased state can lead to neuronal death. Figure 5.3 summarizes the events that may be occurring in a stressed/diseased state, which may contribute to the neuronal death in these states. In both non-stressed and stressed/diseased states, upregulation of system x_c^- leads to increased GSH production and increased glutamate release. In a non-stressed state we hypothesize that increased GSH production helps protect neurons from oxidative stress caused by free radicals and EAATs are able to handle the increased glutamate being released. In a stressed/diseased state, however, increased glutamate release can lead to excitotoxicity due to ionotrophic receptor overactivation. The cause of AMPA and NMDA receptor overactivation is unknown, but may be due to receptor upregulation, recetpor phosphorylation, decreased EAAT function, or a combination of these events. Decreased EAAT function may also decrease the uptake of cysteine into neurons, which would decrease GSH production in neurons leading to increased oxidative stress and contribute to neuronal death.

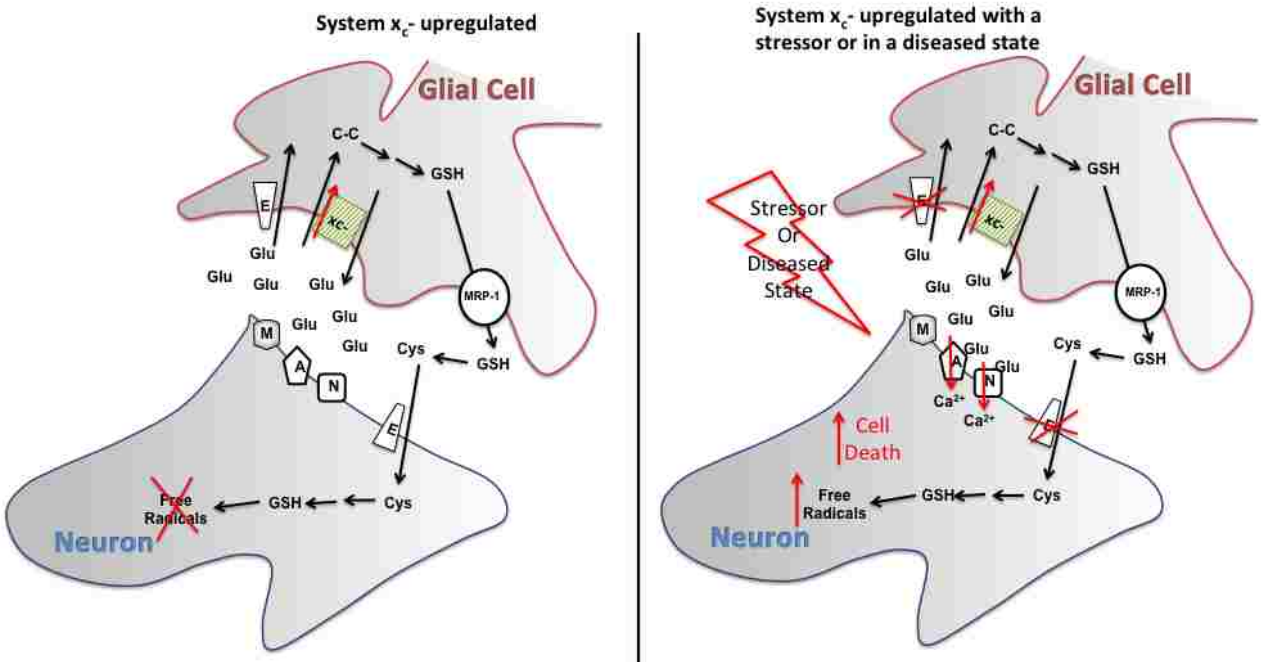

Figure 5.3 Hypothetical role of system x_c^- in contributing to neuronal death in stressed/diseased states. C-C: cystine; Cys: cysteine; GSH: glutathione; Glu: glutamate; xc-: system x_c^-; E: excitatory amino acid transporter; A: AMPA receptor; N: NMDA receptor; M: metabotropic glutamate receptor; MRP-1: multidrug resistance protein 1

LIMITATIONS IN STUDYING SYSTEM X_C^-

Detecting System x_c^-

One of the biggest challenges of studying system x_c^- is detecting its expression and pattern of distribution. Three different xCT transcripts (12, 3.5, and 2.5kb), all easily induced by a variety of insults, have been detected in macrophages, fibroblasts, and HT22 cells using Northern blot (Sato et al., 1999; Sato et al., 2001; Sato et al., 2002; Sato et al., 2004; Sato et al., 2005; Lewerenz et al., 2006). The different transcripts are likely a result of alternative splicing or alternative polyadenylation sites. Northern blot analysis of different types of tissue also show that the 12kb transcript is found in the brain, but is

absent from the heart, lungs, liver, and kidneys. The 3.5 and 2.5kb transcripts are absent from all of the tissue tested, including brain tissue, suggesting that they are mainly found in cultured macrophages (Sato et al., 1999).

Using RT-qPCR, we measured the change in xCT mRNA levels following TGF-β1 application, using two different sets of primers. Both sets of primers showed that TGF-β1 increased xCT mRNA levels in a time-dependent manner to a similar extent (data not shown). Interestingly, our results also showed that the xCT mRNA detected with the first set of primers was significantly more abundant than that detected with the second set of primers under control conditions. These results suggest that we are detecting two different splice variants of xCT.

While using Northern blotting and RT-qPCR are useful, increased xCT mRNA does not always mean increased functional protein at the membrane. A major hurdle for studying system x_c^- is the lack of a specific antibody to detect xCT protein. The amino acid sequence for xCT is predicted to be 502 amino acids long and have a relative molecular weight of 55.5kDa (Sato et al., 1999). However, Western blotting with different antibodies has yielded results with multiple molecular masses. A 50kDa band was detected in human glioma cells (Kim et al., 2001), while a 45kDa band was detected in murine fibroblasts (Lewerenz et al., 2009). In Muller cells, both 50kDa and 40kDa bands were detected (Mysona et al., 2009). The 50kDa band was found both in the membrane and intracellularly. The 40kDa band, on the other hand, was mainly found intracellularly; its expression increased in the membrane when the cells were in conditions of increased oxidative stress. A 40kDa band was also found in human fibroblasts, and neurons and astrocytes cultured from rats (La Bella et al., 2007). In

HT22 cells diethyl maleate increased the intensity of a band detected at 40kDa; and this band was gone following RNAi for xCT (Burdo et al., 2006). Finally, a 35kDa band has been detected in multiple brain regions in mice and rats (Shih et al., 2006; Massie et al., 2008; De Bundel et al., 2011; Liefferinge et al., 2016). Some of these studies also show that the 35kDa band is not present in xCT knockout mice.

A recent study done by Liefferinge et al. (2016) highlights some of the problems regarding xCT antibodies. Out of 53 antibodies studied, only one was specific enough to be present in the xCT $^{+/+}$ mouse and absent in the xCT $^{-/-}$ mouse when used for immunohistochemistry staining. This was an antibody that was made in-house and it showed only one band at 35kDa with Western blotting. Using this one specific antibody they were able to detect xCT expression in the cortex, striatum, hippocampus, midbrain, thalamus, and amygdala. All of the other antibodies analyzed (including commercially available antibodies) detected multiple molecular masses with Western blotting and/or showed non-specific labeling with immunohistochemistry. Liefferinge et al. (2016) also noted that the bands labeled with Western blot could change between different stocks of the same antibody. Due to the unreliability of xCT antibodies, in this thesis we focused on analyzing system x_c- function using radiolabeled uptake assays.

Limitations Regarding the Translation of *in vitro* Studies *in vivo*

In working with primary cortical cultures we have observed that system x_c- is mainly found on astrocytes. There are a couple of key pieces of evidence that support this observation. First, ^{14}C-cystine uptake through system x_c- is much greater under control conditions in astrocytes-enriched glial cortical cultures than in either pure neuronal cultures or pure microglia cultures. Also, when TGF-β1 is applied ^{14}C-cystine

uptake is only increased in astrocyte-enriched glial cultures. Similarly, other studies have found that IL-1β and PACAP increase ^{14}C-cystine uptake and xCT mRNA expression in astrocytes, but not in neuronal or microglial cultures (Jackman et al., 2010; Resch et al., 2014). Second, we observe similar levels of ^{14}C-cystine uptake in mixed cortical cultures, which contain both neurons and glial cells, and mixed cortical cultures treated with 500μM NMDA, which effectively kills the neurons without killing the astrocytes. Taken together, this data suggests that in our culture system, system x_c^- is mainly found and upregulated on astrocytes.

Other *in vitro* studies show that meninges have a higher rate of radiolabeled cystine uptake than astrocytes (Shih et al., 2006) and that system x_c^- is upregulated on microglia (Piani and Fontana, 1994; Qin et al., 2006; Barger et al., 2007). System x_c^- activity has even been induced in culture on cells that do not show xCT expression *in vivo* (Takada and Bannai, 1984; Watanabe and Bannai, 1987), which raises some concerns about translating *in vitro* studies *in vivo*. Culturing conditions likely make cells much more reliant on system x_c^-, which makes its activity much higher *in vitro* than what may be found *in vivo* (Lewerenz et al., 2013). For example, oxygen levels used in culturing conditions are higher than oxygen levels found *in vivo*. Cells are normally cultured under 21% oxygen conditions, which leads to significantly higher levels of cystine uptake than cells cultured under only 2% oxygen (Bannai et al., 1989; Sato et al., 2001). Higher levels of oxygen lead to an increase in ROS (Halliwell, 2003) and in order to be protected, cells must synthesize more GSH. Since cysteine found in the media is rapidly oxidized to cystine under normal cell culture conditions, *in vitro* cells rely heavily

on system x_c- to be able to take up cystine from the media to convert it to GSH (Conrad and Sato, 2012).

Within this thesis there is conflicting data regarding system x_c- activity between work done using *in vitro* spinal cord glial cells cultured from 1-3 day old SOD1-G93A mice pups and work done in *ex vivo* slices from adult SOD1-G93A mice (Chapter II). In order to further study system x_c- in SOD1-G93A mutant mice we cultured spinal cord glial cells from 1-3 day old pups. Consistently culturing and getting healthy cells to grow *in vitro* from SOD1-G93A adult tissue is difficult, therefore it is common to culture cells from young SOD1-G93A mutant pups (Hensley et al., 2006; Bilsland et al., 2008; Cassina et al., 2008; Rabinovich-Toidman et al., 2015). After culturing the glial cultures we insulted them with iron citrate, in order to mimic the increase in oxidative stress seen *in vivo* in SOD1-G93A mutant mice. However, when we did this SOD1-G93A non-transgenic littermates had higher levels of cystine uptake than SOD1-G93A mutant mice, which is the opposite of what we observed in adult spinal cord slices. While *in vitro* studies reveal useful insight into the mechanistic and pharmacological characteristics of system x_c-, the differences we see in system x_c- activity between *in vitro* culture and *ex vivo* slice, along with the concerns regarding culture conditions, must be kept in mind when considering what is learned *in vitro* and translating it *in vivo*.

Limitations of the DCF assay

The DCF assay is the most commonly used assay to detect oxidative stress (Kalyanaraman et al., 2012). In this assay the cell-permeable probe, H_2DCFDA is added to the media. Once inside the cell it is de-esterified to form a free acid (H_2DCF) that can then be oxidized to the fluorescent product, DCF, which is easily detected using a

fluorescent plate reader. Originally thought to mainly detect hydrogen peroxide (H_2O_2) it is now known that H_2DCF can be oxidized to DCF by the hydroxyl radical ($\bullet OH^-$), $\bullet NO_2^-$ formed from the nitrite system, hypochlorous acid (HOCl), and reactive species formed peroxynitrite decomposition (Kalyanaraman et al., 2012).

Although easy to use, in our hands, we found that the DCF assay could give mixed results. As previously discussed, we used the DCF assay to detect the levels of oxidative stress inside astrocyte-enriched glial cell cultures following TGF-β1. Initially we observed an increase in DCF fluorescence following 1-hour TGF-β1 application, which indicated an increase in intracellular oxidative stress. However, in subsequent experiments the increase in oxidative stress was no longer detected by the DCF assay. The mixed results that we observe with the DCF assay may be a result of some of the assay's caveats. One such caveat to the assay that may explain our mixed is that H_2DCF is not directly oxidized by H_2O_2 (Kalyanaraman et al., 2012). A short (30 minute) exposure of cells to H_2O_2 only marginally increased DCF in a study by Karlsson et al. (2010). Therefore, it is possible that if TGF-β1 is increasing system xc- activity by increasing H_2O_2 production, the DCF assay may not be able to reliably detect the increase in H_2O_2 after only 1-hour.

The DCF assay may also have some more major caveats. The study by Karlsson et al. (2010) suggests that H_2DCF oxidation largely occurs as a result of its interaction with cytochrome c, a protein released from the mitochondria during apoptosis, and/or its interaction with iron released from lysosomes, which forms DCF in the presence of oxygen or H_2O_2. The study suggests that without the release of cytochrome c and/or iron only a weak cytosolic fluorescence will be observed, which is likely due to normal

mitochondrial production of H_2O_2. These caveats should also be taken into consideration when interpreting data from the DCF assay.

CONCLUSIONS

The function of system x_c^- allows it to be influential in determining a cell's fate. Its dual nature allows it to protect neurons from oxidative stress but it may also lead to cell death by releasing too much glutamate into the extracellular space. Although discovered over 30 years ago, the question still remains: Is upregulation of system x_c^- more likely to be neuroprotective or lead to cell death? Unfortunately, because of the complexity of system x_c^- function and the diseases it is dysregulated in, there is no clear answer to this question. However, this thesis was able to add to our knowledge about how system x_c^- is regulated and its contribution to cell death.

Our study was the first to observe system x_c^- dysregulation in a model of ALS. We found that system x_c^- activity was increased in SOD1-G93A mutant mice at a time point just after increased oxidative stress was observed. Given its function, this suggests that system x_c^- activity is increased in response to oxidative stress in order to provide antioxidant protection. However, system x_c^- is increased in many neurodegenerative diseases and appears to have negative consequences on neuronal death in many of these diseases. We also found cysteine to be a more important regulator of system x_c^- activity than GSH. One of system x_c^-'s functions is to maintain the cystine/cysteine redox balance of the cellular membrane; therefore, cysteine's ability to regulate system x_c^- is important, especially since levels of cysteine are more directly related to the uptake of cystine than levels of GSH. Finally, we showed that TGF-β1 increased system x_c^-

activity on astrocytes and that this can have negative consequences on neurons when they are under increased oxidative stress.

Collectively, there appear to be more studies showing negative consequences of increased system x_c- function on neuronal death and our TGF-β1 study adds to this collection. However, it is important to point out that most of these observations are seen in culture and occur when another stressor is involved. Increasing system x_c- activity *in vivo* may be beneficial in situations where excitotoxicity is limited, but oxidative stress is occurring. Therapeutically increasing system x_c- activity may also be useful in order to restore impaired glutamatergic signaling, as is seen in diseases like schizophrenia (Baker et al., 2008; Chen et al., 2010; Lutgen et al., 2014) and addiction (Baker et al., 2003; Madayag et al., 2007; Kau et al., 2008; Reichel et al., 2011); in which excitotoxicity is not likely to occur.

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