Characterization of System xc -, a Cystine-Glutamate Amino Acid Transporter, as an Effector of Interleukin-1β-Mediated Injury and Neuroprotection

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Interleukin-1β (IL-1β) is a key mediator in the inflammatory response essential to cellular defense against pathogen invasion and to the repair of tissue damage. While some studies suggest that IL-1β signaling is harmful to the injured central nervous system, others report neuroprotective effects that appear to be context-dependent. Previously, the Hewett laboratory demonstrated the importance of IL-1β-mediated increases in the cystine-glutamate transporter, system \( x_c^- \), as a novel contributor to inflammatory hypoxic neuronal injury, a model of the ischemic penumbra. The main focus of this doctoral research was to elucidate the specific cell type(s) in mixed cortical cultures that respond to IL-1β by enhancing the activity of system \( x_c^- \), the molecular mechanism by which this occurs, and the physiological and pathophysiological consequences of this regulation. IL-1β exposure (0.1-3 ng/ml) enhances expression of the substrate-specific light chain of system \( x_c^- \), xCT, in pure astrocyte cultures in a time-dependent manner. By utilizing pharmacological inhibitors and cells derived from animals harboring a mutation in the \( Slc7a11 \) gene (sut mice) that encodes for xCT, we now provide definitive evidence that alterations in system \( x_c^- \) activity in astrocytes exclusively mediate the potentiation of
hypoxic neuronal injury by IL-1β and initiates hypoglycemic neuronal injury. Additionally, the neuronal cell death in these paradigms is non-cell autonomous. Even though the IL-1β-mediated enhanced efflux of glutamate, which occurs by virtue of its obligate exchange, can be deleterious, cystine import via system x_c is critical for the synthesis of the antioxidant glutathione (GSH). Since astrocytes function as the predominant provider of GSH in the CNS, we assessed whether IL-1β treatment altered astrocyte GSH levels. Pure cortical astrocyte cultures treated with IL-1β (3 ng/ml) exhibit a time-dependent increase in extracellular GSH levels, suggesting both enhanced synthesis and export that is associated with protection against oxidative stress in pure astrocyte cultures as well as mixed cultures exposed to tert-butyl hydroperoxide (tBOOH; 0.1-1.5 mM). Hence, IL-1β may be an important stimulus for increasing astrocytic GSH production, and thus, total antioxidant capacity in the brain. These findings unequivocally demonstrate that IL-1β and astrocytic system x_c contribute to neuronal injury and neuroprotection in a context-dependent manner.
Characterization of System $x_c^-$, a Cystine-Glutamate Amino Acid Transporter, as an Effector of Interleukin-1β-Mediated Injury and Neuroprotection

Nicole Alyse Jackman

B.A., Brown University, 2002

A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

at the University of Connecticut

2013
Characterization of System $x_c^-$, a Cystine-Glutamate Amino Acid Transporter, as an Effector of Interleukin-1β-Mediated Injury and Neuroprotection

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2013
ACKNOWLEDGEMENTS

This work would not have been possible without the support of family, friends, and colleagues. First, it is with immense gratitude that I acknowledge the mentorship, and friendship of my PI, Sandra Hewett who taught me much about science and life. My experience in the Hewett\textsuperscript{2} lab was further enhanced by the mentorship of Jim Hewett. Without his support and efforts, genotyping of the sut mice may not have occurred. I am grateful to Tracy Uliasz for her friendship and my initial training in the lab. Further, lab camaraderie was an important contributor to my success. I thank Rob Claycomb for his insight, humor, and friendship and acknowledge all the others who enhanced my time in lab including, Kumiko Ijichi, Anthony Giampetruzzi, Janna Silakova, Adam van Dyke, and especially two undergraduate students, Shannon Melchoir and Michael Hermelin, who contributed to this work. I am indebted to my committee members, Steve Crocker, Betty Eipper, Jim Hewett, and Louise McCullough for their guidance. Science requires collaboration; thus, I thank Doug Lobner and Travis Rush (Marquette University), Jun Li (University of Connecticut Health Center), Hideyo Sato (Yamagata University), and Jeff Rothestein (Johns Hopkins). Additionally, this thesis would not have been possible without the support of Steve Pfeiffer, the MD/PhD Program and its Program Directors, the Department of Neuroscience, and continued assistance provided by the departmental administrators. I am grateful to the National Institute of Neurological Disorders and Stroke (NINDS) and the National Institute on Aging (NIA) for their financial support of these efforts.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-CPG</td>
<td>(S)-4-carboxyphenylglycine</td>
</tr>
<tr>
<td>4F2hc</td>
<td>heavy chain of the surface antigen 4F2, a.k.a. CD98</td>
</tr>
<tr>
<td>8-Br-cGMP</td>
<td>8-bromo-cyclic GMP</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-hydroxy deoxyguanosine</td>
</tr>
<tr>
<td>AARE</td>
<td>Amino acid responsive element</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid B-peptide</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>a.k.a.</td>
<td>Also known as</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>AraC</td>
<td>β-D-arabinofuranoside</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BGS</td>
<td>bovine growth serum</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
</tr>
<tr>
<td>BSS</td>
<td>balanced salt solution</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>cPLA₂</td>
<td>Cytosolic phospholipase A2</td>
</tr>
<tr>
<td>CS</td>
<td>calf serum</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Cyss</td>
<td>Cystine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dBcAMP</td>
<td>dibutyl cyclic AMP</td>
</tr>
<tr>
<td>ΔΔCT</td>
<td>comparative cycle threshold method for qPCR analysis</td>
</tr>
<tr>
<td>DEM</td>
<td>diethyl maleate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>E14</td>
<td>embryonic day 14</td>
</tr>
<tr>
<td>EAAT</td>
<td>excitatory amino acid transporter, a.k.a. system XAG&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>EAAT1</td>
<td>excitatory amino acid transporter, a.k.a. GLAST</td>
</tr>
<tr>
<td>EAAT2</td>
<td>excitatory amino acid transporter, a.k.a. Glt-1</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EpRE</td>
<td>electrophile response element</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GCL</td>
<td>γ-glutamylcysteiny ligase</td>
</tr>
<tr>
<td>GCLC</td>
<td>γ-glutamylcysteiny ligase catalytic subunit</td>
</tr>
<tr>
<td>GCLM</td>
<td>γ-glutamylcysteiny ligase modifier subunit</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GGT1</td>
<td>γ-glutamyl transpeptidase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GRO-α</td>
<td>Growth regulated oncogene-α a.k.a CXCL1</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSS</td>
<td>glutathione synthetase</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>GSx</td>
<td>total glutathione = (GSH + GSSG)</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1-converting enzyme</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth-1</td>
</tr>
</tbody>
</table>
IκB  inhibitor of NF-κB
IKK  inhibitor of NF-κB kinase
IL-1α  Interleukin-1α
IL-1β  Interleukin-1β
IL-1RI  Interleukin-1 Receptor type I
IL-1RII  Interleukin-1 Receptor type II
IRAK  IL-1R-associated kinase
JNK  c-Jun N-terminal
K_m  substrate concentration at which velocity is ½ of V_max.
KO  knockout
LADMAC  cell line which supplies colony stimulating factor-1
LAT  light chain of the system L amino acid transporter
LDH  lactate dehydrogenase
LDL  low-density lipoprotein
LPS  lipopolysaccharide
LTP  long term potentiation
MAP  mitogen activated protein
MCAO  middle cerebral artery occlusion
MnSOD  manganese superoxide dismutase
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Mrp1  multidrug resistance protein 1
MS  multiple sclerosis or media stock
MTT  thiazolyl blue tetrazolium bromide
MyD88  myeloid differentiation primary response gene 88
NAC  N-acetyl cystine
NF-κB  nuclear factor κB
NGF  nerve growth factor
NMDA  N-methyl-D-aspartic acid
NO  nitric oxide
Nrf2  nuclear factor erythroid derived 2-related protein
NT-3  Neurotrophin-3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>P7</td>
<td>postnatal 7</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-Ribose) polymerase-1</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDTC</td>
<td>pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RBAT</td>
<td>related to the b⁰⁺ amino acid transporter</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>sAPP</td>
<td>Secreted β-amyloid precursor protein</td>
</tr>
<tr>
<td>slc7a11</td>
<td>solute carrier family 7 member 11</td>
</tr>
<tr>
<td>sut</td>
<td>subtle gray mutation (mutation in xCT/slc7a11)</td>
</tr>
<tr>
<td>tat</td>
<td>transactivation of transcription</td>
</tr>
<tr>
<td>TBOA</td>
<td>DL-threo-β-Benzylxyaspartic acid</td>
</tr>
<tr>
<td>tBOOH</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-IL-1 receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tollip</td>
<td>Toll-interacting protein</td>
</tr>
<tr>
<td>TRAF6</td>
<td>tumor necrosis factor receptor associated kinase</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vₘₐₓ</td>
<td>rate of transport at maximal concentrations of substrate</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>xCT</td>
<td>light subunit of system x&lt;sub&gt;c&lt;/sub&gt; transporter</td>
</tr>
</tbody>
</table>
CHAPTER 1

General principles
1.1 Introduction

Inflammation is an intrinsically complex biological response mounted to combat pathogen invasion, protect against tissue damage and promote tissue repair. However, unabated inflammation can be deleterious and contribute to injury and pathology. Given that inflammation is a fundamentally protective response, it should not be surprising that inflammatory mediators can exert beneficial effects. This dissertation focuses specifically on the pleiotropic cytokine, interleukin-1β (IL-1β) described by some as “the master regulator of neuroinflammation” (Basu et al., 2004), and the ability of IL-1β to contribute to neuronal injury under conditions of energy deprivation and protect neurons against oxidative stress-induced injury via its regulation of an amino acid exchanger known as system x_c which transports the amino acids cystine and glutamate.

1.2 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS (Fonnum, 1984). It is involved in developmental processes such as cell migration and differentiation, and also in most physiological processes in the brain including learning and memory via its interaction with AMPA and NMDA receptors, and energy metabolism, by serving as a precursor to α-ketoglutarate. Regulated control of glutamate release and efficient uptake of this transmitter are required.
for glutamatergic signaling under physiological conditions. In the event of dramatic enhancements in extracellular glutamate, the prolonged stimulation of glutamate receptors can initiate cell death via a process termed excitotoxicity (see section 1.2.4).

1.2.1 Glutamate Physiology

Glutamatergic neurons make up 90% of all neurons in the brain (Dingledine and McBain, 1999). In this subset of neurons, glutamate is packaged in synaptic vesicles and released via exocytosis upon depolarization of the nerve terminals. Once released, glutamate either binds to its receptors, diffuses away from the synapse, or is cleared from the synapse via excitatory amino acid transporters (EAAT1-5 also known as system X$_{AG}^{-}$) which are localized to both neurons and astrocytes. Notably, astrocytic glutamate uptake via EAAT1/GLAST and EAAT2/Glt-1 is the predominant route of glutamate clearance, with Glt-1 responsible for clearance of 80% of glutamate in the hippocampus (Lehre and Danbolt, 1998). Glutamate that is internalized via astrocytic transporters can be converted to glutamine via the action of glutamine synthetase, an enzyme localized exclusively to astrocytes (Norenberg and Martinez-Hernandez, 1979). Conversion of glutamate to glutamine enables synaptically released glutamate to be recycled and shuttled back to neurons in a non-neuroactive form. Within the neuron glutamine is converted back to glutamate via glutaminase. Alternatively, glutamate can be converted to α-ketoglutarate, a key intermediate in the Kreb's cycle, via the enzymatic action of glutamate dehydrogenase or glutamate oxaloacetate transaminase.
1.2.2 Glutamate Signaling

Glutamate binds to two distinct classes of receptors, ionotropic and metabotropic receptors. Ionotropic receptors flux cations following glutamate binding, whereas metabotropic receptors activate intracellular signaling via G-protein-coupled second messenger systems. There are three types of ionotropic receptors named according to agonist pharmacology: the N-methyl-D-aspartate (NMDA) receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptor, and the kainate receptor. Additionally there are 8 metabotropic receptors which are separated into three groups based on sequence homology, second messenger systems, and pharmacological agonist and antagonist profiles. Group I metabotropic glutamate receptors consist of mGluR1 and mGluR5 and typically activate phospholipase C pathways and increase intracellular Ca\(^{2+}\). Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, and mGluR8) metabotropic receptors generally reduce adenylate cyclase activity [for a detailed review see (Nakanishi et al., 1998; Dingledine et al., 1999; Featherstone and Shippy, 2008; Kim et al., 2008)].

1.2.3 Basal ambient extracellular glutamate

At its most basic level, ambient glutamate levels are dictated by the rate of release and the rate of glutamate clearance from the extracellular space. To fully appreciate ambient extracellular glutamate levels, one must first understand glutamate compartmentalization within the CNS. Neurons and astrocytes have
millimolar concentrations of intracellular glutamate, with neurons having higher levels of cytosolic glutamate due to the action of glutamine synthetase in astrocytes which converts glutamate to glutamine (Nedergaard et al., 2002). The concentration of glutamate in the cytosol of glutamatergic neurons can reach 10 mM, with an estimated glutamate concentration of up to 100 mM in the synaptic vesicles (Riveros et al., 1986; Burger et al., 1989; Clements et al., 1992; Ottersen et al., 1992; Danbolt, 2001). Extracellular glutamate levels in the synapse fluctuate as a function of neuronal activity and range from 2 µM – 1 mM (Meldrum, 2000; Danbolt, 2001).

Numerous techniques have been employed to estimate extracellular glutamate levels in the brain with concentrations estimated to range between ≈25 nM to 5 µM (Danbolt, 2001; Baker et al., 2002b; Baker et al., 2003; Cavelier and Attwell, 2005; Nyitrai et al., 2006; Herman and Jahr, 2007) depending on the particular area analyzed and technique employed. For example, in vivo microdialysis measurements of extracellular glutamate levels range from 1-2 µM in the striatum (Rothstein et al., 1996; Baker et al., 2002b) to 5 µM in rat nucleus accumbens (Baker et al., 2002b; Baker et al., 2003). Importantly, micromolar concentrations of glutamate could activate glutamate receptors as the EC$_{50}$ of the NMDA receptor for glutamate is 2 µM (Patneau and Mayer, 1990; Herman and Jahr, 2007). AMPA/kainate receptors are activated by glutamate at much higher concentrations and thus are not likely to be activated by ambient levels of extracellular glutamate (for EC$_{50}$ values for other glutamate receptors see Table 1). Alternatively, micromolar concentrations of glutamate could cause receptor
desensitization and the suppression of glutamate signaling. Sather and colleagues desensitized ≈80% of NMDA receptors with 1 µM glutamate in outside-out patches from embryonic mouse neurons (Sather et al., 1992). AMPA/kainate receptors desensitize at concentrations ranging from 3 – 13 µM (Featherstone and Shippy, 2008), making it possible that some subset of ionotropic glutamate transporters are tonically desensitized by ambient glutamate.

It is possible that microdialysis studies overestimate extracellular glutamate concentrations as the insertion of the microdialysis probe damages tissue, creates an artificial space surrounding the probe and in turn increases the distance between the probe and functional transporters (Benveniste and Huttemeier, 1990). Herman and Jahr measured currents produced by application of NMDA and glutamate in rat CA1 pyramidal neuronal slices and estimated a value of ≈25 nM ambient extracellular glutamate (Herman and Jahr, 2007). Since glutamate is rapidly taken up by the EAATs, the EC$_{50}$ values obtained from dose-response curves for NMDA and glutamate were compared to create a conversion factor which was utilized to estimate ambient glutamate. The authors contend that ambient glutamate levels maintained at nanomolar concentrations would prevent NMDA receptor activation / desensitization (Herman and Jahr, 2007).
Table 1. ED$_{50}$ values for glutamate activation of glutamate receptors. Values obtained from (Conn and Pin, 1997; Meldrum, 2000; Featherstone and Shippy, 2008).

<table>
<thead>
<tr>
<th>Receptors</th>
<th>ED$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>2-3</td>
</tr>
<tr>
<td>AMPA</td>
<td>200-500</td>
</tr>
<tr>
<td>Kainate</td>
<td>300-800</td>
</tr>
<tr>
<td>mGluR1</td>
<td>9-13</td>
</tr>
<tr>
<td>mGluR2</td>
<td>4-20</td>
</tr>
<tr>
<td>mGluR3</td>
<td>4-5</td>
</tr>
<tr>
<td>mGluR4</td>
<td>3-20</td>
</tr>
<tr>
<td>mGluR5</td>
<td>3-10</td>
</tr>
<tr>
<td>mGluR6</td>
<td>16</td>
</tr>
<tr>
<td>mGluR7</td>
<td>1000</td>
</tr>
<tr>
<td>mGluR8</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Based on analysis of the stoichiometric transport of the anionic form of glutamate, 3 Na$^+$, and 1 H$^+$ and the counter transport of 1 K$^+$ in EAAT3 transfected oocytes, EAAT / system X$_{AG}^-$ transporters are predicted to have the capacity to theoretically reduce extracellular glutamate to $\approx$ 2 nM (Zerangue and Kavanaugh, 1996). Yet, only one study has calculated extracellular glutamate to lie near this theoretical lower limit (Herman and Jahr, 2007). Naturally, it raises the question of the true concentration of ambient glutamate and the source(s) maintaining glutamate levels in the extracellular compartment.

Inhibition of glutamate uptake by the application of DL-threo-beta-benzyloxyaspartate (TBOA) in organotypic hippocampal slices results in
increased extracellular glutamate. This glutamate is not synaptically released, and is not dependent on Na\(^+\) and Ca\(^{2+}\) (Jabaudon et al., 1999). Further, increasing levels of astrocytic intracellular glutamate via inhibition of glutamine synthetase enhances glutamate accumulation following inhibition of glutamate uptake suggesting a glial source (Jabaudon et al., 1999).

Application of cystine (1 mM) to cerebellar and cortical slices produces an inward, glutamate dependent current in Purkinje and pyramidal cells and the hypothesis that a cystine-glutamate exchanger regulates extracellular glutamate levels emerged (Warr et al., 1999). Additional supportive evidence was noted in microdialysis studies in the striatum. Reverse dialysis of a cystine-glutamate transporter antagonist reduces extracellular glutamate by 60% (Baker et al., 2002b). Further, the function of this transporter is conserved across species as genetic disruption of the same cystine-glutamate transporter via insertion of a mutation in the first exon of the genderblind (gb) gene in \textit{Drosophila} produces a 50% reduction in extracellular glutamate (Augustin et al., 2007).

Importantly, basal extracellular glutamate levels dictate the density of post-synaptic ionotropic glutamate receptor clustering in \textit{Drosophila}. Mutant genderblind \textit{Drosophila} with reduced ambient glutamate levels have increased glutamate receptor immunoreactivity and enhanced spontaneous excitatory currents at the neuromuscular junction (Augustin et al., 2007). Glutamate receptor density was restored with physiological levels of glutamate (Augustin et al., 2007). Additionally, ambient basal extracellular glutamate tonically activates mGluR2/3 receptors to negatively modulate glutamatergic and dopaminergic
signaling in the striatum and nucleus accumbens (Baker et al., 2002b; Moran et al., 2005). Interestingly, cocaine administration reduces extracellular glutamate in the rat nucleus accumbens and this alteration in glutamatergic tone is associated with drug-seeking behavior (i.e. susceptibility to relapse) following cocaine withdrawal (Baker et al., 2002b; Kalivas, 2009).

1.2.4 Glutamate Excitotoxicity

Extracellular glutamate levels must be carefully regulated as prolonged stimulation of its cognate receptors will lead to neuronal cell death. The concept of excitotoxicity is described as the excessive and toxic activation of excitatory amino acid receptors that culminates in cell death (Olney, 1969; Choi, 1992). Normally, glutamate concentrations in the extracellular space are maintained at low levels, however, if levels rise dramatically as a result of increased release or an impairment in the clearance of glutamate, a calcium-dependent cascade is initiated (Sattler and Tymianski, 2001). Ultimately, the prolonged activation of glutamate receptors enhances Na\(^+\) and Ca\(^{2+}\) flux into the cells (Figure 1.1). The ensuing Ca\(^{2+}\) dysregulation activates kinases, proteases, and lipases, and increases the production of reactive oxygen and nitrogen species which impairs mitochondrial function and results in cell death [reviewed extensively in (Choi, 1988; Sattler and Tymianski, 2000, 2001)].
Figure 1.1. **Mechanisms of excitotoxicity.** High levels of glutamate activate the AMPA receptor, inducing sodium (and calcium) influx and neuronal depolarization. Depolarization induces the release of Mg$^{2+}$ from the NMDA receptor which is activated by the binding of glutamate and its co-agonist glycine to flux sodium and calcium. Voltage-dependent calcium channels (VDCC) are also activated. Intracellular calcium dramatically increases and activates proteases, phospholipases, and nucleases, and induces the production of free radicals and reactive species. Ultimately the cascade leads to cell death. Modified from (Camacho and Massieu, 2006).
1.2.6 Sources of extracellular glutamate

Glutamate originating in neurons can be released via exocytosis following depolarization or as a consequence of cell lysis. Glutamate is released from astrocytes via Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent processes (Parpura et al., 1994; Araque et al., 1998; Jabaudon et al., 1999). Astrocytes express several proteins required for exocytosis including synaptobrevin II and syntaxin (Parpura et al., 1994; Parpura et al., 1995; Araque et al., 1998), and ligands such as prostaglandin E\(_2\), ATP, glutamate, and bradykinin are known to induce glutamate release (Parpura et al., 1994; Bezzi et al., 1998; Jeremic et al., 2001). Further, the reverse operation of glutamate transporters (system X\(_{AG^-}\)) could enhance extracellular glutamate levels (Nicholls and Attwell, 1990; Szatkowski et al., 1990; Longuemare and Swanson, 1995), however this likely only operates under pathological conditions when the Na\(^+\) and K\(^+\) gradient across the plasma membrane is reduced such as occurs under cerebral ischemic conditions. Glutamate efflux can arise as a consequence of activation of volume sensitive organic anion channels as a component of volume regulation (Kimelberg et al., 1990; Longuemare et al., 1999; Liu et al., 2006). Interestingly, inhibition of a VSOAC with 5-nitro-2-3-phenylpropylamino benzoate (NPPB) can attenuate receptor-mediated glutamate release from astrocytes (Jeremic et al., 2001). Other sources of glutamate release include: P2X7 channels (Duan et al., 2003), hemichannels (Ye et al., 2003; Takeuchi et al., 2006), and system x\(_{c^-}\) (Warr et al., 1999), however this dissertation is particularly interested in release of glutamate via system x\(_{c^-}\), and as such will focus on the cystine-glutamate exchanger as the
primary source of the excitotoxic levels of extracellular glutamate observed in the paradigms described herein.

1.3 System $x_c^-$

System $x_c^-$ was first identified in 1980 in the IMR-90 human fibroblast cell line as a sodium-independent electroneutral transporter of the amino acids cystine and glutamate (Bannai and Kitamura, 1980). Cystine and glutamate both significantly decrease transport of each other, whereas minimal inhibition of cystine and glutamate transport by other amino acids is observed, demonstrating the specificity of the transporter. The $K_m$ for cystine is reported as 40-50 µM in fibroblasts (Bannai and Kitamura, 1980) which is consistent with the value of $\approx 30$ µM obtained for cystine in the mixed cortical cultures routinely used in our laboratory (Fogal et al., 2007). The import of cystine via system $x_c^-$ is directly coupled to glutamate export, occurring in a Na$^+$-independent, Cl$^-$-dependent manner with 1:1 stoichiometry (Bannai, 1986). Further, transport is limited to the anionic forms of these amino acids with exchange being electroneutral.

System $x_c^-$ is a member of the heteromeric amino acid transporter family in which transporters consist of a heavy and light subunit joined via an extracellular disulfide bond (for a detailed review see (Chillaron et al., 2001)). With respect to system $x_c^-$, xCT is the light chain that confers substrate specificity, and 4F2hc (heavy chain of the 4F2 surface antigen also known as CD98) is the heavy chain that is thought to target the transporter to the plasma membrane (Sato et al.,
1999; Bassi et al., 2001). Alternatively, xCT can interact with RBAT (related to the $b^{o+}$ amino acid transporter), an alternate heavy chain to produce a functional transporter in experimental systems in which the cDNA of both subunits is injected into *Xenopus* oocytes (Bassi et al., 2001). However, it remains to be determined if this interaction occurs naturally *in vivo*. 
Figure 1.2. Structure of system $x_c^-$ amino acid transporter. System $x_c^-$ is a heteromeric amino acid transport system consisting of two subunits: xCT (white) -- the light chain that confers substrate specificity -- and a heavy chain (4F2hc depicted; black) or RBAT -- that is thought to target the transporter to the plasma membrane (Sato et al., 1999; Bassi et al., 2001). The subunits are joined via an extracellular disulfide bond. The import of cystine via system $x_c^-$ is directly coupled with glutamate export, occurring in a Na$^+$-independent, Cl$^-$-dependent manner with 1:1 stoichiometry (Bannai, 1986). Adapted from (Danbolt, 2001; Shih et al., 2006).
1.3.1 Structure of system $x_c^-$

The light subunit of system $x_c^-$, xCT, was first identified and cloned by Sato and colleagues in 1999 from mouse peritoneal macrophage cells (Sato et al., 1999). Later, human xCT was identified (Sato et al., 2000; Bassi et al., 2001; Kim et al., 2001) with full length human xCT cloned from an undifferentiated human teratocarcinoma cell line. The human xCT gene, also known as solute carrier family 7 member 11 ($Slc7a11$), is located on chromosome 4q28-31, and produces a 501 amino acid protein which shares 89% sequence identity and 93-96% similarity with the 502 amino acid-containing mouse xCT (Bassi et al., 2001; Bridges et al., 2001). An additional truncated xCT clone thought to be a splice variant has been observed, but its function remains to be determined (Sato et al., 2000; Kim et al., 2001). As shown in Figure 1.2, xCT has 12 putative membrane spanning domains, with both the N- and C- termini localized intracellularly (Gasol et al., 2004). Structural data remains elusive with respect to the location of substrate binding. Gasol and colleagues believe that substrate binding occurs within loops 2 and 3 as biotinylation of His$^{110}$ does not occur in the presence of the system $x_c^-$ substrates cystine and glutamate, or the non-transportable system $x_c^-$ inhibitor (S)-4-carboxyphenylglycine (4-CPG) (Gasol et al., 2004). The same group found that the 8th putative transmembrane domain close to Cys$^{327}$ also participates in substrate binding and/or permeation (Jimenez-Vidal et al., 2004). Traditionally, the light subunits of the Slc7 family are not glycosylated, although species-specific differences in glycosylation are reported as human xCT possesses a site for N-linked glycosylation (Asn$^{314}$) in the extracellular loop.
between transmembrane domains 7 and 8, whereas mouse-derived xCT does not (Bridges et al., 2001).

4F2hc (also known as CD98) is a type II glycoprotein with a single transmembrane domain, a molecular weight of ≈ 85-kDa (Jimenez-Vidal et al., 2004; Palacin and Kanai, 2004). Importantly, the 4F2hc subunit is thought to be in excess compared to xCT and, as such, need not be dynamically regulated like its light chain partners (Nakamura et al., 1999; Verrey et al., 2000). 4F2hc is widely expressed and the tissue and cell-type distribution demonstrate its importance in a myriad of biological activities. xCT and 4F2hc interact via an extracellular disulfide bond at residue Cys$^{158}$ of xCT (Gasol et al., 2004). The heavy chain of system $x_c^-$, 4F2hc, interacts with numerous other amino acid transporter light chains via a disulfide bridge and functions to traffic the heterodimer to the plasma membrane (Nakamura et al., 1999). Other roles for 4F2hc have been described such as involvement in cell adhesion/polarity and functioning as an extracellular signaling molecule (Nguyen et al., 2008; Yan et al., 2008). In transfected Xenopus oocytes, it has been eloquently demonstrated that both the light and heavy chains of system $x_c^-$ are required for transporter function (i.e. injection of xCT alone or 4F2hc alone cannot produce functional cystine uptake, whereas co-injection can) (Sato et al., 1999). Similar findings are observed with human xCT. However, mouse xCT can interact with either heavy chain 4F2hc or RBAT and have functional transporter activity as assessed by cystine uptake (Wang et al., 2003), whereas co-expression of human xCT with human RBAT fails to produce functional transporter activity (Bassi et al., 2001).
1.3.2 System $x_c$ expression

System $x_c$ is widely distributed in a variety of tissues and is almost ubiquitously expressed in cultured mammalian cells. xCT mRNA and/or protein is present in the brain, liver, kidney, stomach, intestines, ovary, spleen, and numerous other organs and cell types (Lo et al., 2008). Within the CNS, system $x_c$ is expressed in neurons (Murphy et al., 1990; Burdo et al., 2006; Dun et al., 2006), astrocytes (Bender et al., 2000; Gochenauer and Robinson, 2001; Pow, 2001; Burdo et al., 2006), microglia (Piani and Fontana, 1994), and glioma cell lines (Cho and Bannai, 1990; Ye et al., 1999). Further, system $x_c$ is present at the CNS-periphery interface and highly expressed in vascular endothelial cells, meningeal cells, and ependymal cells (Burdo et al., 2006) and in situ hybridization studies show strong expression of xCT mRNA in the area postrema, subfornical organ, hypothalamus and the meninges (Sato et al., 2002).

1.3.3 System $x_c$ function: Glutathione biosynthesis

In addition to its role in regulating ambient extracellular glutamate, system $x_c$ has traditionally been studied with respect to its involvement in the biosynthesis of the antioxidant molecule glutathione (GSH; Figure 1.3). In fact, in early studies performed by Bannai and Kitamura in IMR-90 cells, approximately 60% of radiolabeled cystine transported into the cells via system $x_c$ is reduced to cysteine within five minutes, while 15% of imported cystine is rapidly incorporated into GSH in the same time frame (Bannai and Kitamura, 1980). Thus, the function of system $x_c$ is intimately related to intracellular glutathione levels.
Similar findings have demonstrated that enhanced activity of system $x_c^-$ increases intracellular GSH in an endothelial cell line (MBEC4), a rat retinal Müller cell line (TR-MUL), and in BHK21 cells derived from the Syrian hamster kidney (Hosoya et al., 2002; Sasaki et al., 2002; Tomi et al., 2003). Conversely, in numerous neural cell types, including neurons, gliomas, and oligodendrocyte precursors, inhibition of cystine transport in with system $x_c^-$ antagonists such as glutamate, homocysteic acid, and quisqualate results in diminished intracellular stores of GSH and subsequent cell death (Murphy et al., 1989; Murphy et al., 1990; Kato et al., 1992; Oka et al., 1993; Sagara et al., 1993; Ratan et al., 1994; Back et al., 1998; Chung et al., 2005).

GSH is a peptide composed of glutamate, cysteine, and glycine, and is the most abundant non-protein thiol in mammalian cells. GSH is best known as a potent antioxidant molecule that directly detoxifies free radicals, but also serves as a cofactor for the antioxidant enzyme glutathione peroxidase which neutralizes hydrogen peroxide and other organic peroxide species. GSH also plays important roles in cell metabolism and proliferation, gene expression, and signal transduction (Poot et al., 1995; Dringen, 2000; Wu et al., 2004; Shih et al., 2006). Glutathione also participates in signal transduction by modifying redox-sensitive proteins in the face of alteration in oxidative and/or nitrosative homeostasis (Stamler and Hausladen, 1998; Klatt and Lamas, 2000). Hence, understanding GSH synthesis, metabolism, and regulation is of great import.

Given the inability of GSH to cross the BBB (Cornford et al., 1978), de novo GSH synthesis within the CNS is essential. The concentration of cysteine in
the extracellular space is low as its disulfide bond is rapidly oxidized in this compartment, thus intracellular GSH concentrations are critically dependent on cystine, the best extracellular precursor for cysteine. Numerous amino acid transporters have the ability to import cystine (Kakuda and MacLeod, 1994; Knickelbein et al., 1997), however, in the CNS, cystine is predominantly transported via system \( x_c \) which is the focus of this dissertation (Bender et al., 2000; Shanker and Aschner, 2001; McBean, 2002).

GSH synthesis occurs in the cytosol via a two-step enzymatic reaction, catalyzed by \( \gamma \)-glutamylcysteine ligase and glutathione synthetase, with cysteine as the rate-limiting substrate (Meister and Anderson, 1983; Deneke and Fanburg, 1989; Sagara et al., 1993; Kranich et al., 1996). Once produced glutathione can be exported from the cell, cleaved by \( \gamma \)-glutamyltranspeptidase into the \( \gamma \)-glutamyl moiety and a cysteinylglycine dipeptide, which can be further catabolized via the action of aminopeptidase N (Dringen et al., 2001).

The concentration of GSH is estimated to range from 1-3 mM in the CNS and approaches 8 mM in rat astrocyte primary cultures (Dringen and Hamprecht, 1998; Dringen, 2000; Franco et al., 2008). Since astrocytes function as indispensible support cells for neurons, it may not be surprising that astrocytes possess higher GSH levels than neurons (Desagher et al., 1996; Wang and Cynader, 2000), and protect neurons against \( \text{H}_2\text{O}_2 \), dopamine, and NO-mediated oxidative insults (Desagher et al., 1996; Tanaka et al., 1999; Chen et al., 2001; Shih et al., 2003; Jakel et al., 2007). In fact, 10% of astrocytic GSH / hour is exported to the extracellular compartment (Sagara et al., 1996; Minich et al.,
2006), with the multidrug resistant protein (Mrp1) transporting approximately 60% of this GSH released from astrocytes as determined using a competitive Mrp1 inhibitor, MK-571 (Hirrlinger et al., 2002). Importantly, export of GSH and its extracellular catabolism provides GSH precursors which are utilized for GSH biosynthesis in neurons. As a result of neuronal dependence on astrocytic GSH, deficiencies in astrocytic GSH render neurons more susceptible to oxidant-induced injury (Gegg et al., 2005), presumably due to reduced export of GSH which negatively influences neuronal GSH synthesis (Raps et al., 1989; Sagara et al., 1993; Kranich et al., 1996; Dringen et al., 1999; Minich et al., 2006; Sun et al., 2006).
Figure 1.3. Schematic of glutathione biosynthesis and metabolism. The structure of reduced glutathione (GSH) is depicted. Modified from (Owen and Butterfield, 2010).
1.3.4 System $x_c^-$ and disease

The link between system $x_c^-$ activity and CNS pathology in vivo has been extensively studied in the context of the biology of gliomas. System $x_c^-$ is expressed in rodent and human glioma cell lines and patient-derived gliomas (Ye et al., 1999; Ye and Sontheimer, 1999; Chung et al., 2005; Lyons et al., 2007; Ogunrinu and Sontheimer, 2010). Interestingly, the activity of system $x_c^-$ facilitates tumor growth and expansion within the confines of the skull via glutamate-mediated excitotoxic neuronal cell death. In fact, glioma cultures can raise extracellular glutamate concentrations to 500 µM within 12 hr (Ye and Sontheimer, 1999). This in vitro finding was substantiated in vivo by the finding that implantation of glioma cells in rats could produce substantial increases in extracellular glutamate as measured via microdialysis (Behrens et al., 2000). Further, there is evidence that disruption of system $x_c^-$ via siRNA-mediated gene knockdown of xCT reduces glioma-derived glutamate release and reduces edema and neuronal injury (Savaskan et al., 2008). Additionally, in vitro studies implicate system $x_c^-$ activity as contributory to injury in a variety of disease states including cerebral ischemia (Fogal et al., 2007), Alzheimer’s disease (Barger and Basile, 2001; Qin et al., 2006), and multiple sclerosis (Domercq et al., 2007). Further, new in vivo studies note enhanced expression of system $x_c^-$ following experimental autoimmune encephalomyelitis (EAE) and speculate a role for system $x_c^-$ in demyelinating diseases as oligodendrocytes are also susceptible to glutamate excitotoxicity (Pampliega et al., 2011).
1.3.5 Animal models deficient in system $x_c^{-}$ function

The subtle gray (sut) mouse arose via a natural mutation in the slc7a11 gene which encodes for xCT, the light chain of system $x_c^{-}$. Sut mice possess a large $\approx 500,000$ base deletion that extends from intron 11 through exon 12 and into the intergenic region adjacent to the Pcdh18 gene (Chintala et al., 2005). As a result of this mutation, no xCT protein is produced and the mice have a subtle coat color phenotype secondary to the inability to create pheomelanin, a red/yellow pigment produced by the addition of cysteine to dopaquinone (Chintala et al., 2005). In particular, there is a reduction in the intensity of yellow pigment in agouti mice such that the coat color is gray, rather than brown with yellow highlights. In addition to alterations in coat color, these mutant animals also have prolonged bleeding times which are associated with abnormalities in platelet dense granules (Swank et al., 1996). Sut mice show evidence of neurodegeneration that is apparent by 13 weeks of age and is characterized by ventricular enlargement, striatal atrophy, and cortical thinning (Shih et al., 2006).

In 2005, Sato and colleagues generated an xCT knock-out (KO) mouse where the xCT gene was disrupted in all cell types via deletion of the translation initiation site and exon one (Sato et al., 2005). No abnormalities are noted in any of the organs examined in 8 week old mice including the brain, lung, liver, pancreas, and kidney. It raises the possibility that the neurodegeneration observed in sut/sut mice may arise due to the C3H/HeSnJ background, and not as a function of the loss of xCT gene function. xCT KO mice are viable, fertile, and healthy in appearance at 6 months. Interestingly, plasma cystine
concentrations in the xCT KO mice are double the concentrations seen in WT mice and there are no other differences in amino acid concentrations. The plasma concentration of GSH in KO mice is half the concentration detected in WT mice. However, there are no genotype-specific differences in GSH content in the liver or brain. Remarkably, adaptive compensatory responses must have been initiated to allow survival of xCT-null animals, because fibroblasts, melanocytes, and astrocytes derived from xCT mutants [both xCT KO mice and the spontaneous (sut/sut) mutant mice] fail to thrive in culture in the absence of a reducing agent such as β-mercaptoethanol (Appendix Figure A1), however, viability can be rescued with the addition of a reducing agent, such as β-mercaptoethanol, in the tissue culture media (Chintala et al., 2005; Sato et al., 2005; Shih et al., 2006).

Of note, while system $\mathbf{x}_c$ is often studied in the context of its contributions to glutathione synthesis, closer examination of the transporter shows that its role as a regulator of extracellular glutamate levels is likely more important \textit{in vivo} as loss of transporter function dramatic alters extracellular glutamate levels with no effects of tissue glutathione in the striatum or hippocampus (Massie et al., 2010b; De Bundel et al., 2011). Irrespective of which biological function is qualitatively more important, alterations in system $\mathbf{x}_c$ activity and/or expression is associated with pathology thus it is important to understand how this transporter is regulated.
1.3.6 Regulators of system $x_c^-$ activity

The activity of system $x_c^-$ can be dynamically regulated in both positive and negative fashions. For instance, cellular deprivation of several amino acids (e.g. cystine, arginine, and tryptophan) or exposure to lipopolysaccharide (LPS), nitric oxide (NO), dibutyryl cAMP (dBcAMP), or electrophilic reagents such as diethyl maleate (DEM), increase xCT expression and/or system $x_c^-$ activity (Bannai and Kitamura, 1982; Bannai, 1984a; Kim et al., 1987; Miura et al., 1992; Sato et al., 1999; Bridges et al., 2001; Gochenauer and Robinson, 2001; Kim et al., 2001; Sato et al., 2001; Nosoya et al., 2002; Tomi et al., 2002; Sato et al., 2004).

In the CNS, secreted amyloid precursor protein (sAPP), amyloid β-peptide (Aβ), chronic dBcAMP treatment, and TNF-α, but not IL-1β, can enhance system $x_c^-$ activity in microglial cells (Piani and Fontana, 1994; Barger and Basile, 2001; Qin et al., 2006; Seib et al., in press). Chronic treatment of astrocytes with dibutyryl cAMP (dBcAMP) enhances the activity of system $x_c^-$ (Gochenauer and Robinson, 2001). Previous data from our laboratory indicate that the cytokine IL-1β mediates an enhancement of system $x_c^-$ activity in mixed cortical cell cultures predominately containing neurons and astrocytes, although cell-type specificity was not determined in these studies (Fogal et al., 2007).
1.4.1 Interleukin-1β (IL-1β)

IL-1β is a member of the canonical interleukin-1 (IL-1) family that consists of two agonists IL-1α and IL-1β, an endogenous antagonist IL-1ra, and two receptors (IL-1RI and IL-1RII). Currently there are 11 known members of the IL-1 family with additional members added to the family based on conservation of key amino acid sequences and similar three-dimensional structures [reviewed by (Dinarello, 2009; Sims and Smith, 2010)]. Further discussion will be limited to IL-1β, the key cytokine discussed in this thesis. [For a more detailed review of other IL-1 family members see (Arend et al., 2008; O'Neill, 2008; Sims and Smith, 2010)].

IL-1β is a pleiotropic cytokine with a variety of effects on numerous cell types. Although IL-1 was the first member of the group of peptides known as cytokines to be described, it was originally known by more colorful and functionally descriptive names that demonstrate the role of IL-1β in the inflammatory response, such as lymphocyte activating factor, B-cell differentiation factor, and endogenous pyrogen, among others (Dinarello, 1988). The last name listed, endogenous pyrogen, highlights the role of IL-1β as a mediator which influences cells within the immune system but also within the central nervous system (CNS). Specifically, IL-1β exerts its effects at the level of hypothalamus to alter body temperature via prostaglandin-mediated induction of fever [thoroughly reviewed in (Dinarello et al., 1999; Dinarello, 2004)].

The gene encoding IL-1β is located on chromosome 2 in humans and mice (Webb et al., 1986) and IL-1β is generated as a 31-kDa precursor protein
referred to as pro-IL-1β. Pro-IL-1β is inactive until cleaved by the intracellular cysteine protease, caspase-1/ interleukin-1-converting enzyme (ICE), which produces the mature, functionally active 17-kDa protein (Jobling et al., 1988; Howard et al., 1991). First, there is a priming step where IL-1β mRNA expression is enhanced without a concomitant increase in IL-1β protein (Schindler et al., 1990), whereby various stimuli, such as the complement component C5a, hypoxia, or adherence of monocytes to glass or plastic surfaces induces large amounts of IL-1β mRNA without significant translation into protein (Haskill et al., 1988; Ghezzi et al., 1991; Dinarello, 1998). Without a signal for translation, IL-1β is rapidly degraded with a half-life ranging from 2 – 4.5 hr (Schindler et al., 1990). Importantly, mature protein is only detected extracellularly suggesting that the processing of pro-IL-1β is temporally coordinated with IL-1β release (Hogquist et al., 1991).

Importantly, the IL-1β precursor protein lacks an N-terminal hydrophobic signal denoting a cleavage site which would target it for processing by the endoplasmic reticulum (ER)/Golgi apparatus (Gray et al., 1986) and subsequent secretion from the cell. In the absence of such a signaling sequence, immunoelectron microscopy utilizing an antibody against human IL-1β demonstrates accumulation of the cytokine within the cytoplasm, whereas none is detected in the ER/Golgi complex or in secretory vesicles of human monocytes (Singer et al., 1988). Thus, a classical pathway of exocytosis is not utilized for IL-1β release.
Some consensus has been reached in that many agree that IL-1β secretion is dependent on ATP, its purinergic receptor P2X7 (Sanz and Di Virgilio, 2000; Solle et al., 2001; Bianco et al., 2005; Clark et al., 2010), and calcium (MacKenzie et al., 2001; Andrei et al., 2004). Importantly, macrophages lacking P2X7 receptors fail to secrete IL-1β following ATP exposure, but secretion induced by the K+ ionophore, nigericin, is not altered in the P2X7-deficient cells suggesting the involvement of an additional regulatory pathway(s) (Solle et al., 2001). Additionally, IL-1β is not secreted from lumbar spinal cord slices derived from P2X7-null mice or in the presence of a P2X7 receptor antagonist (Clark et al., 2010). There are several schools of thought as to how IL-1β is released (Figure 1.4). The most parsimonious model of IL-1β release is that it is transported through uncharacterized transporters located in the plasma membrane (Brough and Rothwell, 2007). MacKenzie and colleagues propose a model of release dependent on ATP-induced shedding of IL-1β-containing microvesicles within seconds of P2X7 receptor activation (MacKenzie et al., 2001). This is similar to a proposed mechanism where exosome-containing multivesicular bodies release IL-1β containing exosomes into the extracellular space (Qu et al., 2007). However, the mechanism by which IL-1β traverses the microvesicle membrane remains elusive. Qu and colleagues have speculated that microvesicle membranes, like the plasma membrane, also contain P2X7 receptors which initiate microvesicle lysis in the presence of ATP (Bianco et al., 2005) or that vesicle lysis is mediated by the action of phospholipases and proteases from the secretory lysosome (Qu et al., 2007). Notably, Qu and
colleagues were unable to detect IL-1β-containing microvesicles in the tissue culture supernatant. Andrei and colleagues present an alternate model where ATP-mediated depletion of intracellular $K^+$ activates caspase-1 and phosphatidylcholine-specific phospholipase C. Ultimately, the activation of phosphatidylcholine-specific phospholipase C and cytosolic phospholipase A2 (cPLA$_2$) trigger lysosome exocytosis and the release of IL-1β, caspase-1, and other lysosomal enzymes (Andrei et al., 2004). Finally, a novel caspase-1 dependent form of cell death called pyroptosis has been hypothesized to participate in release of IL-1β via plasma membrane pores under ischemic conditions and after infection (Bergsbaken et al., 2009). Of note, a study of diarylsulfonurea-mediated inhibition of cytokine release from human mononuclear cells (irrespective of the initiating stimuli) demonstrates that glutathione S-transferase may be involved in the release of IL-1β into the extracellular space (Laliberte et al., 2003). Further study of the mechanism of release is warranted, as the full picture remains elusive.
Figure 1.4. Four models of non-classical IL-1β secretion. Pathway 1 depicts the direct efflux of cytosolic IL-1β (and other inflammasome components) through uncharacterized transporters. Pathway 2 depicts microvesicular shedding / blebbing. Pathway 3 depicts Ca\(^{2+}\)-dependent secretory lysosome exocytosis. Pathway 4 depicts the multivesicular bodies (MVB) / exosome model. Modified from (Qu et al., 2007).
1.4.2 IL-1 Signaling

Indisputably, IL-1β bind to two receptors, the type I or type II receptor referred to as IL-1RI or IL1-RII, respectively (Sims et al., 1988). Both the IL-1RI and IL1-RII receptors are located on chromosome 2 in humans and chromosome 1 in mice (Mantovani et al., 1998). These two receptors are similar in that both receptors possess three extracellular immunoglobin-like domains, however the cytosolic domains differ greatly. IL-1RI possesses an ≈ 200 amino acid intracellular domain whereas the intracellular domain of IL-1RII consists of ≈ 29 amino acids (Dinarello, 1991). As one might expect, receptor function varies as a consequence of the structural differences in the cytosolic domain. A long intracellular domain allows IL1-RI to signal (Sims et al., 1993), whereas the absence of a signaling domain in IL1-RII renders it a “decoy receptor”, thought to serve as a negative regulator (McMahan et al., 1991; Colotta et al., 1994).

IL-1RI signaling culminates in the activation of several signaling pathways including the nuclear factor-κB (NF-κB) pathway, the p38 mitogen activated protein (MAP) kinase pathway, and the c-Jun N-terminal kinase (JNK) pathway (for a more detailed review see (O'Neill and Greene, 1998; Ichijo, 1999; Dunne and O'Neill, 2003)). Since IL-1β preferentially activates NF-κB in astrocytes (Srinivasan et al., 2004), the classical activation of NF-κB will be the only pathway discussed and is as follows: IL-1β binds to IL-1RI which induces a conformational change in IL-1RI (Vigers et al., 1997) facilitating the interaction with its accessory protein, IL-1RAcP (Greenfeder et al., 1995). The interaction between IL-1RI and the accessory protein recruits the adaptor protein myeloid
The IL-1 system is tightly regulated and inhibition of IL-1β-mediated signaling can be accomplished via IL-1ra occupation of the IL-1β binding site on IL-1RI. Importantly, the endogenous antagonist IL-1ra cannot induce the conformational change in IL-1RI required to promote an interaction with the accessory protein IL-1RαCp (Greenfeder et al., 1995). Additionally, the decoy receptor IL1-RII competes with IL1-RI for IL-1β binding (Colotta et al., 1993; Re et al., 1994). IL1-RII can interact with the accessory protein, IL-1AcP which would interfere with the normal IL-1RI / IL-1AcP complex formation (Lang et al., 1998; Wang et al., 2010). Finally, both IL1-RI and IL1-RII can be shed from the membrane to sequester ligands (Penton-Rol et al., 1999; Orlando et al., 2000).
Of note, there are three studies that propose the existence of an additional IL-1 responsive signaling receptor in the CNS that remains to be conclusively identified (Parker et al., 2002; Touzani et al., 2002; Andre et al., 2006). Touzani and colleagues demonstrate that IL-1β enhances injury in IL-1RI-null mice following middle cerebral artery occlusion (MCAO) and that injury is not attenuated by the concomitant administration of IL-1β and its endogenous antagonist IL-1ra (Touzani et al., 2002). Furthermore, Andre and colleagues found that IL-1β increases the expression of approximately 400 genes in mixed glial cultures derived from IL-1RI-null mice compared to vehicle treated mixed glial cultures (that have alterations in the expression of almost 1300 genes in response to IL-1β) (Andre et al., 2006). Importantly, contaminants such as endotoxin in the recombinant IL-1β preparation could produce identical results in an IL-1RI-independent manner, however this possibility was not discussed.

1.4.3 IL-1β and CNS physiology

IL-1β has important functions within a normal, but immunologically challenged organism, and its role in the regulation of temperature and sickness behavior, and in the initiation of the inflammatory response has been extensively studied and will not be discussed here [for a detailed review see (Dinarello, 2004; Dantzer and Kelley, 2007)]. Since cytokines function as intercellular messages, additional functions of IL-1β outside the traditional realm of inflammation have been reported. IL-1β induces changes that may protect neurons and promote growth and/or survival via stimulated production of neurotrophic factors,
chemokines and cytokines, and their receptors including GRO-α, tumor necrosis factor (TNF-α), IL-6, and the growth factors nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), and granulocyte colony-stimulating factor (G-CSF) (Gadient et al., 1990; Albrecht et al., 2002; Meeuwsen et al., 2003; John et al., 2005).

Long-term potentiation is defined as a “form of synaptic plasticity which is characterized by a persistent increase in synaptic efficacy following tetanic stimulation of an afferent pathway. On the basis of its properties, LTP has been proposed as a biological substrate for learning and memory” (Lynch, 1998). Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) demonstrates that IL-1β expression is enhanced one hour after LTP induction in rat hippocampal slices, and similar findings are observed in vivo in the ipsalateral hippocampus of rats that had robust potentiation lasting at least 8 hr (Schneider et al., 1998). IL-1RI knockout mice have deficits in hippocampal dependent spatial learning suggesting that endogenous IL-1β may promote LTP and learning (Avital et al., 2003) This is particularly interesting in light of the finding that TNFα, a cytokine induced by IL-1β, promotes astrocytic glutamate release by regulating the number of readily releasable glutamatergic vesicles adjacent to the plasma membrane (Santello et al., 2011) Others have found that IL-1ra has no effect on the induction of LTP, whereas administration of IL-1ra after LTP induction impairs the maintenance phase (Schneider et al., 1998; Coogan et al., 1999). Additionally, lipopolysaccharide (LPS), a potent inducer of IL-1β
expression, can produce learning and memory deficits (Tanaka et al., 2006). While LPS-treatment was associated with an increase in IL-1β as assessed via immunohistochemistry, it remains to be ascertained whether this phenomenon is IL-1β-dependent, which have been hypothesized to occur via inhibition of Ca²⁺ currents in hippocampal neurons (Plata-Salaman and Ffrench-Mullen, 1992, 1994), or via the enhancement of GABA currents (Miller et al., 1991). In sum, endogenously produced IL-1β appears necessary for the expression and maintenance of LTP in hippocampal slices (Schneider et al., 1998; Avital et al., 2003). In contrast, higher concentrations of IL-1β applied exogenously or induced via LPS, which mimic pathological IL-1β expression, inhibit LTP (Bellinger et al., 1993; Cunningham et al., 1996; Murray and Lynch, 1998; Coogan et al., 1999; Avital et al., 2003; Ross et al., 2003).

1.4.4 IL-1β and CNS disease

There is a place for IL-1β in normal physiology, however, dramatic deviations from low levels of basal expression are thought to underlie the ability of IL-1β to facilitate injury. IL-1β is dramatically upregulated in a variety of acute and chronic neurological diseases and disorders [for a thorough review see (Allan et al., 2005; Gosselin and Rivest, 2007; Fogal and Hewett, 2008)]. The strongest evidence for the participation of IL-1β in neuronal injury in a disease state stems from experimental ischemia models. Like numerous other neurological diseases and disorders, IL-1β mRNA and protein are increased after transient and permanent middle cerebral artery occlusion (MCAO) in rodents.
Minami et al., 1992; Liu et al., 1993; Buttini et al., 1994; Sairanen et al., 1997; Davies et al., 1999; Pearson et al., 1999). Yamasaki demonstrated that intraventricular injection of IL-1β enhances neuronal injury in rats following MCAO (Yamasaki et al., 1995). In contrast, intracerebroventricular administration and genetic overexpression of IL-1ra, the endogenous antagonist, before MCAO, is protective and reduces infarct size (Relton and Rothwell, 1992; Betz et al., 1995; Loddick and Rothwell, 1996; Yang et al., 1997; Mulcahy et al., 2003). Finally, animals lacking both IL-1α and IL-1β (but not either ligand alone) (Boutin et al., 2001; Ohtaki et al., 2003), the signaling receptor IL-1RI (Basu et al., 2005; Fogal et al., 2007), or ICE/capase-1, the enzyme required for IL-1β processing (Hara et al., 1997a; Hara et al., 1997b; Schielke et al., 1998; Liu et al., 1999), are protected against hypoxic/ischemic injury. Additionally, administration of an IL-1β neutralizing antibody improves outcomes following ischemia in rats (Yamasaki et al., 1995).

From a clinical research perspective, IL-1β levels in humans increase following cerebral ischemia in a manner consistent with findings from experimental studies. The concentration of IL-1β measured in the CSF of stroke patients is increased 2 days post stroke, which then declines over time to control values, with no observable alterations in plasma IL-1β concentration (Griffin et al., 1994; Tarkowski et al., 1995). Impressively, clinical trials have demonstrated that intravenous administration of recombinant human IL-1ra can penetrate the blood brain barrier (BBB) following stroke and improve clinical outcomes three months post-stroke (Emsley et al., 2005).
Our laboratory is interested in understanding the mechanism by which IL-1β contributes to neuronal injury following cerebral ischemia. As such, we have developed an *in vitro* model of the metabolically active, yet functionally impaired tissue surrounding the ischemic core, which is referred to as the ischemic penumbra. Using *in vitro* cortical preparations we have devised a model that recapitulates the injury that is observed in the ischemic penumbra. Notably, treatment of cultures with exogenous IL-1β, to mimic release from microglia and deprivation of oxygen leads to a potentiation of neuronal cell death (Fogal et al., 2005a; Fogal et al., 2007). Injury can be blocked with the endogenous antagonist, IL-1ra or a IL-1β blocking antibody. Injury is dependent on glutamate-mediated excitotoxicity, astrocytes, and extracellular cystine. As such, we identified system $x_c^-$ as a target of IL-1β whereby inhibition of this transporter attenuates injury (Fogal et al., 2007).

While the deleterious role of IL-1β in ischemia is clear, similar to the dichotomy observed with system $x_c^-$, enhanced levels of IL-1β need not contribute to injury in all disorders. In fact, elevations in IL-1β expression may function to promote tissue repair. Mice lacking IL-1β show reduced remyelination in a cuprizone model of demyelination, with the inability to properly remyelinate occurring secondary to a reduction in insulin-like growth factor-1 (IGF-1) (Mason et al., 2001). Further support of the beneficial role of IL-1β comes from the finding that chronic overexpression of IL-1β reduces pathology in a mouse model of AD (Shaftel et al., 2007). Interestingly, mice deficient in IL-1RI had less neuronal sprouting and functional recovery after 6-hydroxydopamine-mediated injury to the
substantia nigra pars compacta, compared to their wild-type counterparts (Parish et al., 2002). Importantly, in vitro findings further support the assertion that IL-1β can promote positive outcomes by reducing excitotoxic neuronal cell death induced by the ionotropic glutamate receptor agonists NMDA, AMPA, and kainate (Strijbos and Rothwell, 1995; Ohtsuki et al., 1996; Wang et al., 2000; Pringle et al., 2001; Bernardino et al., 2005).

1.5 Significance

A variety of neurological diseases and disorders are associated with excitotoxicity, oxidative stress, and enhanced expression of IL-1β (Fogal and Hewett, 2008) and system x_c⁻ unites all these components. Although system x_c⁻ is best known for its role in the synthesis of the antioxidant molecule GSH (Watanabe and Bannai, 1987; Bannai et al., 1989; Miura et al., 1992; Sato et al., 1995a; Bridges et al., 2001; Dun et al., 2006; Lewerenz et al., 2009a), enhanced transporter activity and subsequent efflux of glutamate in the CNS has been demonstrated to contribute to neuronal and oligodendrocyte injury both in vitro and in vivo (Barger and Basile, 2001; Chung et al., 2005; Qin et al., 2006; Domercq et al., 2007; Fogal et al., 2007; Savaskan et al., 2008; Sontheimer, 2008). Hence, the activity of system x_c⁻ must be tightly controlled. However, it is plausible that enhanced activity of the transporter may arise as a protective mechanism to limit oxidative stress — and excitotoxicity is the unfortunate consequence of a protective response gone awry. Understanding the regulation
of system $x_c^-$ will be required to devise therapies to increase system $x_c^-$ and intracellular GSH to reduce oxidative injury, and when appropriate, to employ strategies to reduce its activity to decrease the probability of excitotoxic neuronal injury.
1.6 Specific Aims

Because enhanced activity of system \( x_c^- \) can facilitate excitotoxic injury or promote the synthesis of the antioxidant glutathione depending on the context, it is important to understand how the transporter is regulated. Our laboratory has demonstrated that the cytokine interleukin-1β (IL-1β) — which is upregulated in numerous neurological diseases/disorders — enhances the activity of the amino acid transporter system \( x_c^- \), mediating an increase in extracellular glutamate and in cellular cyst(e)ine, a constituent of the tripeptide antioxidant molecule glutathione (GSH). Thus, the objective of this dissertation was to elucidate the cellular, molecular, and biochemical mechanisms by which IL-1β regulates system \( x_c^- \). Further, the functional consequences of system \( x_c^- \) activity under pathophysiological and physiological conditions were evaluated.

1.6.1 Specific Aim #1: To determine the cell type(s) responsible for enhanced system \( x_c^- \) activity in response to IL-1β.

The Hewett laboratory previously demonstrated that interleukin-1β (IL-1β) increases system \( x_c^- \) (cystine/glutamate antiporter) activity in mixed cortical cell cultures containing neurons and astrocytes in a manner dependent on astrocyte IL-1RI activation (Fogal et al., 2007), yet the specific cell type(s) responding to IL-1β via an enhancement in system \( x_c^- \) activity was not ascertained. Thus the goal of this specific aim was to identify which specific cell type(s) responds to IL-1β by
enhancing the expression and functional activity of system $x_c^{-}$ by exposing purified populations of neurons, astrocytes, and microglia to IL-1$\beta$ (Chapter 2).

1.6.2 Specific Aim #2: To elucidate the consequences of system $x_c^{-}$ activity under pathological (Chapter 3) and physiological (Chapter 4) conditions.

The Hewett lab previously demonstrated that IL-1$\beta$ potentiates hypoxic and hypoglycemic neuronal injury (Fogal et al., 2005a). The IL-1$\beta$-mediated enhancement in system $x_c^{-}$ activity in mixed cortical cell cultures resulted in a potentiation of hypoxic neuronal injury when glutamate clearance was impaired (Fogal et al., 2007). Despite longstanding evidence that hypoglycemic neuronal injury is also mediated by glutamate excitotoxicity, the role of system $x_c^{-}$ in this paradigm had not been assessed. Specifically, mixed cortical cell cultures were deprived of glucose for up to 8 hr and pharmacological and genetic techniques were employed to assess the involvement of system $x_c^{-}$ in hypoglycemia-induced neuronal injury.

Additionally, the enhancement in system $x_c^{-}$ activity by IL-1$\beta$ under physiological (normoxic and normoglycemic) conditions was assessed by treating astrocytes with IL-1$\beta$ and measuring intracellular and extracellular glutathione levels. Other cellular proteins participating in GSH biosynthesis, export, and metabolism in response to IL-1$\beta$ were also evaluated to see if IL-1$\beta$ alters their expression. Finally, we assessed if the IL-1$\beta$-mediated enhancement of system $x_c^{-}$ activity could confer protection against an oxidative stressor.
CHAPTER 2

Regulation of system $x_c$ activity and expression in astrocytes by interleukin-1β: implications for hypoxic neuronal injury

This chapter is a near duplicate of the previously published work:

2.1 Summary

We recently demonstrated that interleukin-1β (IL-1β) increases system x_c^- (cystine/glutamate antiporter) activity in mixed cortical cell cultures, resulting in an increase in hypoxic neuronal injury when glutamate clearance is impaired. Herein, we demonstrate that neurons, astrocytes and microglia all express system x_c^- subunits (xCT, 4F2hc, RBAT) and are capable of cystine import. However, IL-1β stimulation increases mRNA for xCT—the light chain that confers substrate specificity—in astrocytes only; an effect blocked by the transcriptional inhibitor actinomycin D. Additionally, only astrocytes show an increase in cystine uptake following IL-1β exposure; an effect associated with a change in xCT protein. The increase in cystine uptake that follows IL-1β is lacking in astrocytes derived from mice harboring a mutation in Slc7a11 (sut gene), which encodes for xCT, and in wild-type astrocytes treated with the protein synthesis inhibitor cycloheximide. IL-1β does not regulate the light chain of the amino acid transporter, LAT2, or the expression and function of astrocytic excitatory amino acid transporters (EAATs), demonstrating some target selectivity. Finally, the enhanced neuronal vulnerability to hypoxia that followed IL-1β treatment in our mixed culture system was not observed in chimeric cultures consisting of wild-type neurons plated on top of sut astrocytes. Nor was it observed in wild-type cultures treated with a system x_c^- inhibitor or an NMDA receptor antagonist. Overall, our data demonstrate that IL-1β selectively regulates system x_c^- activity in astrocytes and that this change is specifically
responsible for the deleterious, excitotoxic effects of IL-1β found under hypoxic conditions.

2.2 Introduction

System $x_c^-$ is a heteromeric amino acid transporter consisting of two subunits: xCT — the light chain that confers substrate specificity — and a heavy chain (4F2hc or RBAT) thought to target the transporter to the plasma membrane (Sato et al., 1999; Bassi et al., 2001). The import of cystine via system $x_c^-$ is directly coupled to glutamate export, occurring in a Na$^+$-independent, Cl$^-$-dependent manner with 1:1 stoichiometry (Bannai, 1986; Reichelt et al., 1997). Although, system $x_c^-$ is best known for its role in the synthesis of the antioxidant molecule glutathione (GSH) (Watanabe and Bannai, 1987; Bannai et al., 1989; Miura et al., 1992; Sato et al., 1995a; Bridges et al., 2001; Dun et al., 2006; Lewerenz et al., 2009a), enhanced transporter activity has been reported to contribute to neuronal and oligodendrocyte injury both in vitro and in vivo (Barger and Basile, 2001; Chung et al., 2005; Qin et al., 2006; Domercq et al., 2007; Fogal et al., 2007; Savaskan et al., 2008; Sontheimer, 2008).

System $x_c^-$ subunits and activity have been demonstrated to be dynamically regulated. For instance, xCT expression and/or the activity of system $x_c^-$ is enhanced following deprivation of certain cellular amino acids or exposure to lipopolysaccharide (LPS), nitric oxide (NO), dibutyryl cAMP (dBcAMP), or to electrophilic reagents such as diethyl maleate (DEM) (Miura et al., 1992; Bridges
et al., 2001; Gochenauer and Robinson, 2001; Sato et al., 2004). Recently, we
demonstrated that the cytokine, IL-1β, enhances system $x_c^-$ activity (i.e.
increases $V_{\text{max}}$) in a mixed cortical cell culture system (Fogal et al., 2007). While
increased activity is not toxic alone, presumably because system $X_{AG}^-$ (glutamate
transport) is sufficient to prevent the toxic accumulation of extracellular glutamate
– under conditions where glutamate uptake is compromised (i.e., hypoxia), this
IL-1β-mediated enhancement of system $x_c^-$ activity contributed to an
enhancement of extracellular glutamate levels, which resulted in excitotoxic
neuronal cell death (Fogal et al., 2005a; Fogal et al., 2007). Although we
previously determined that this enhancement in hypoxic neuronal injury in mixed
cortical cell culture was dependent on astrocyte IL-1RI signaling, the cell type
that demonstrated an increase in transporter activity was not ascertained (Fogal
et al., 2007). As our cultures contain predominantly neurons and astrocytes with
some contaminating microglia, the cellular and molecular target of system $x_c^-$
enhancement by IL-1β was examined herein using purified populations of
primary astrocyte, neuron, and microglial cultures. Results indicate that IL-1β
regulates the expression and activity of system $x_c^-$ in astrocytes exclusively and
that glutamate released via astrocytic system $x_c^-$ directly underlies the neurotoxic
propensity of IL-1β under hypoxic conditions.

Part of the work has been published in abstract form (Jackman et al., 2009;
Jackman and Hewett, 2009).
2.3 Materials and Methods

2.3.1 Cell culture

Cell culture media and experimental buffer compositions were as follows: 

*Media stock (MS):* L-glutamine-free modified Eagle’s medium (Earl’s salt; MediaTech) supplemented with L-glutamine, glucose, and sodium bicarbonate to a final concentration of 2.0, 25.7, and 28.2 mM, respectively; *Glial plating media:* MS containing 10% fetal bovine serum (FBS; Hyclone) and 10% calf serum (CS; Hyclone), 10 ng/ml epidermal growth factor (Invitrogen), 50 IU penicillin, and 50 \( \mu g/ml \) streptomycin (Gibco/BRL); *Neuronal plating media:* Neurobasal media containing 1x B27 (Invitrogen), 2 mM L-glutamine, 50 IU penicillin and 50 \( \mu g/ml \) streptomycin; *Mixed culture plating media:* MS containing 5% CS, 5% bovine growth serum (BGS, Hyclone), 50 IU penicillin, and 50 \( \mu g/ml \) streptomycin. *Microglia growth media:* DMEM (high glucose; Gibco) containing 5% FBS, 2mM L-glutamine, 50 IU penicillin, 50 \( \mu g/ml \) streptomycin and 50% LADMAC (ATCC) -conditioned media to supply colony stimulating factor-1 (Sklar et al., 1985). To produce LADMAC conditioned media, the LADMAC cell line (CRL-2420, ATCC,) was grown to confluence in DMEM containing 5% FBS, 2 mM L-glutamine, 50 IU penicillin and 50 \( \mu g/ml \) streptomycin in 75 cm\(^2\) flasks for ~14 days followed by harvesting, centrifugation (720 \( \times \) g; 3 min) and filtering of the culture supernatant, which is then stored at -80°C. *Maintenance media:* MS containing 10% CS and 50 IU penicillin/ 50 \( \mu g/ml \) streptomycin; *HBSS (mM):* 120 NaCl, 5.4 KCl, 0.8 MgCl\(_2\), 1.8 CaCl\(_2\), 15 glucose, 20 HEPES, 10 NaOH, and 0.01 glycine (pH 7.4). *Balanced Salt Solution [BSS (mM)]:* 116 NaCl, 5.4 KCl, 0.8 MgCl\(_2\), 1 NaH\(_2\)PO\(_4\),
26.2 NaHCO$_3$, 1.8 CaCl$_2$, 0.01 glycine, 2 L-glutamine, 1x MEM amino acids (Invitrogen), and 5 or 20 mM glucose (BSS$_5$ or BSS$_{20}$, respectively).

*Primary astrocyte cultures* were derived from pooled cortices of day 1-3 postnatal CD1 mouse pups (Charles River Laboratories) or from mouse pups derived from *sut* heterozygous breeding pairs (JAX; Stock # 001310) or *sut* control mice (C3H/HeSnJ; JAX; Stock # 000661) essentially as described (Trackey et al., 2001). Additionally, wild-type (WT) and *sut* (xCT-deficient) astrocytes were cultured from cortices of single pups derived from *sut/+* breeding pairs (JAX; Stock # 001310). β-mercaptoethanol (β-ME; 55 µM) was added to the glial plating medium of *sut* cultures to support growth and to WT cultures for control purposes (Shih et al., 2006). The rest of the brain was used for genotyping: WT primers (230 bp product) 5′- GAA GTG CTC CGT GAA GAA GG 3′ (forward), 5′- ATC TCA ATC CTG GGC AGA TG-3′ (reverse); *sut* primers (≈2280 bp product) 5′-CCA CTG TTG TAG GTC AGC TTA GG-3′ (forward), 5′- CAG GAC CTG TGA ATA TGA TAG GG 3′ (reverse). *Il1r1* wild-type and null mutant astrocytes were cultured from cerebral cortices of single pups derived from *Il1r1* heterozygous breeding pairs (JAX; stock # 003245). Following dissection of the cerebral cortices, the rest of the brain was used for genotyping as described [http://jaxmice.jax.org/strain/003245.html]. Cells from cerebral cortices were dissociated, plated (Falcon Primaria; BD Biosciences) and once confluent, then treated with 8 µM β-D-arabinofuranoside (AraC; Sigma) once for 4-7 days to reduce the number of microglia. *Purified astrocyte cultures* were generated by removing residual microglia by treatment with 75 mM L-leucine
methyl ester for 60-90 min, one day prior to experimentation (Hamby et al., 2006a). Cultures were ≤ 35 days in vitro at the time of experimentation. Microglia cultures were prepared by plating dissociated cortical cells from CD1 mouse pups (1-3 days) in T25 tissue culture flasks (2 hemisphere/5 ml/flask) in glial plating medium. Fourteen to 21 days later, the culture medium was supplemented with HEPES buffer to a final concentration of 25 mM and flasks shaken overnight at 150 rpm (37°C). The supernatant containing dislodged microglia was collected, spun (3 min; 720 x g) and the resulting pellet resuspended in microglial growth media and plated in 15 mm 24-well plates (Corning) (Hamby et al., 2006b). Primary neuronal cultures were derived from dissociated cortical cells of embryonic day 15 CD1 mouse fetuses using a modification of the protocol of Brewer and colleagues (Brewer et al., 1993). Two days after plating in neuronal plating medium, cultures were treated once with 1 µM AraC for two days, then media was partially replenished (½ volume exchange) twice weekly. Experiments were performed on pure neuronal cultures after 7-10 days in vitro. Mixed cortical cell cultures containing predominantly neurons and astrocytes with a small amount of contaminating microglia were prepared by isolating cortices obtained from embryonic day 15 mouse fetuses and plating them on a confluent layer of astrocytes in mixed culture plating media (Trackey et al., 2001). After 7 days in vitro, mixed cultures were treated with 8 µM AraC for 2 days then switched into maintenance media. The media was changed after 5 and 9 days in vitro, and one day prior to experimentation, cells were placed into MS. Experiments were performed on mixed cortical cultures after 13-
14 days in vitro. All cultures were maintained at 37°C in a humidified 6% CO₂-containing atmosphere.

2.3.2 IL-1β Treatment

Cells were treated with 3 ng/ml recombinant murine IL-1β (R&D Systems) for various times in an incubation buffer of MS (neurons and astrocytes) or microglial growth media (microglia) both supplemented with 0.1% fatty-acid free BSA (Sigma). Cells were then returned to a humidified 37°C normoxic (21% O₂) incubator containing 6% CO₂.

2.3.3 Radiolabeled L-cystine and D-aspartate uptake

System xc⁻ specific ¹⁴C-L-cystine (PerkinElmer) and system X₅G⁻-mediated ³H-D-aspartate (PerkinElmer) uptake was performed as previously described (Fogal et al., 2007). Cultures were washed into HBSS (3 x 750 µL) and allowed to equilibrate for 10 min (25°C). For cystine uptake, cells were incubated in HBSS containing 3 µM ¹⁴C-L-cystine (1 µCi/ml), 27 µM unlabeled cystine, 1mM D-aspartate and 0.5 mM acivicin (Biomol). D-aspartate and acivicin were included in the uptake buffer to block system X₅G⁻ and γ-glutamyltranspeptidase, respectively. Uptake was terminated by washing in ice-cold PBS (3 x 750 µL). For D-aspartate uptake, cells were incubated in HBSS containing 0.1 µCi/ml ³H-D-aspartate and varying concentrations of unlabeled D-aspartate (25°C) for 5 min and uptake terminated by washing cells with an ice-cold Na⁺-free choline stop
buffer containing in mM: 116 choline chloride, 0.8 MgSO₄, 1 KH₂PO₄, 10 HEPES, 5 KOH, 10 glucose, 0.9 CaCl₂, and 5 non-radioactive D-aspartate. Varying concentrations of unlabeled D-aspartate were used to span the $K_m$ of the different astrocytic glutamate transporters (Danbolt, 2001), ensuring that the concentration of non-radioactive D-aspartate used was not rate-limiting. Cells were lysed with warm 0.5% SDS and accumulated radioactivity estimated using a liquid scintillation counter. Uptake was normalized to total protein as determined by the BCA Assay (Pierce).

2.3.4 Reverse transcriptase-PCR analysis

Three or four wells of cells grown in 24 well tissue culture plates were combined, the RNA was extracted (TRIzol, Invitrogen) and then suspended in 20 μL RNase-free water. First-strand cDNA was synthesized from 0.5-1 μg RNA using M-MLV reverse transcriptase (400 U, Invitrogen) and oligo (d)T primers (Promega) as previously described (Hewett et al., 1999). Reactions were performed in 20 μL volumes at 40-42°C for 1 hr. Each RNA sample was incubated similarly in the absence of reverse transcriptase to test for genomic DNA contamination (none detected). PCR amplimer pairs for analysis of specific cDNAs are as follows:
PCR was performed on 1 µL of cDNA using Taq DNA polymerase (1 U, Invitrogen) in a total volume of 25 µL. Each cycle consisted of a denaturation step (94 °C; 30 sec), an annealing step (45 sec), and a primer extension step (72 °C, 1 min). PCR products were separated by electrophoresis in 2% agarose with 1 kb size markers (Invitrogen) and visualized by ethidium bromide (BioRad) using a UV transilluminator.

2.3.5 Quantitative Real-time PCR (qPCR)

RNA was isolated and first-strand cDNA synthesized as described above. qPCR was performed using mouse-specific primer pairs [Taqman Gene Expression Assays, Applied Biosystems: xCT (Mm00442530_m1); 4F2hc (Mm00500521_m1); RBAT (Mm00486218_m1); EAAT-1 (Mm00600697_m1); EAAT-2 (Mm00441457_m1); LAT2 (Mm00444250_m1)] per manufacturer’s instructions. Reactions were run in the Applied Biosystems Fast Real-Time PCR System and relative quantification performed using the comparative cycle

<table>
<thead>
<tr>
<th>Amplimers</th>
<th>Annealing Temp.</th>
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<tbody>
<tr>
<td>xCT</td>
<td>63 °C</td>
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<td>4F2hc</td>
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<td>RBAT</td>
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<td>β-actin</td>
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Table 2.1- PCR amplimer pairs for RT-PCR.
threshold method ($\Delta \Delta C_T$), where $C_T$ values of the transcript of interest were normalized to $\beta$-actin $C_T$ values from the same sample, then compared to a calibrator $C_T$ value (untreated cells) to determine the relative fold increase in mRNA. $\beta$-actin $C_T$ values were unaffected by IL-1$\beta$ treatment. Statistics were performed on the logarithmic retransformation (i.e. geometric means) of $2^{-\Delta \Delta C_T}$ values. Preliminary experiments were performed to establish that the amplification efficiency for each of the primer pairs was >94%.

### 2.3.6 Immunoblotting

Protein expression was determined by Western Blot analysis. Astrocytes in 24-well plates were washed twice with ice-cold PBS then incubated in 50 µL lysis buffer [50 mM Tris, pH 8.0, 1.0% Nonidet-P40 (NP40), 150 mM NaCl, and protease inhibitor cocktail (Roche)] for 30 minutes on ice. Cells were harvested by scraping, lysates from eight wells pooled, cellular debris removed by centrifugation (10,000 x $g$; 15 min; 4°C), and the resulting supernatants stored at -20°C. After thawing, proteins were concentrated via ethanol precipitation. The pelleted protein was resuspended in 1x urea buffer (50 mM Tris, pH 6.8, 2.5% glycerol, 5% SDS, 4 M Urea, 10 mM DTT, 0.02% bromophenol blue). One hundred µg protein (BCA assay; Pierce) was separated by 10% SDS-PAGE under reducing conditions and electrophoretically transferred to a nitrocellulose membrane (0.2 µm; Bio-Rad). Proteins of interest were detected sequentially using species-specific Western Breeze Immunodetection kits (Invitrogen) per manufacturer’s instructions. Primary antibodies and incubation times were as
follows: xCT (2 µg/ml; rabbit polyclonal; Novus Biologicals; 2 h at 37° C), β-actin (0.3 µg/ml; mouse monoclonal; Sigma; 2 h at 37° C) or CD98 (4F2hc; 0.4 µg/ml; goat polyclonal; Santa Cruz; overnight at 4° C). Results were recorded on X-ray film (FujiFilm). Digitized images were analyzed by computer-assisted densitometry (Gel-Pro Analyzer) and xCT and 4F2hc protein normalized to their respective β-actin levels.

2.3.7 Hypoxia

Mixed cortical cell cultures were placed into an anaerobic chamber (Thermolabs) containing a gas mixture of 5% CO₂, 10% H₂, and 85% N₂ (<0.2% O₂). Culture medium was replaced by thorough exchange with a deoxygenated balanced salt solution (BSS₂). Cells were placed in a 37°C incubator within the chamber for 4-5 hr and then returned to a 37°C, 6% CO₂-containing normoxic (21% O₂) incubator. Parallel cultures within the same plate were placed into deoxygenated BSS₂₀ to assess for neuronal injury unrelated to the experimental paradigm. Importantly, in the presence of 20 mM glucose, cells can resist neuronal injury for nearly 12 hr of oxygen deprivation (Fogal et al., 2005b). Neuronal cell death was assessed 20-24 hr later.

2.3.8 Measurement of neuronal cell death

Neuronal cell death was quantitatively determined by measurement of lactate dehydrogenase (LDH) released into the culture supernatant as described previously (Uliasz and Hewett, 2000). Data are expressed as a percentage of
total neuronal LDH activity (defined as 100%), determined by assaying the supernatant of parallel cultures exposed to 200 µM NMDA for 20-24 hr. Since primary cortical astrocytes do not contain NMDA receptors (Backus et al., 1989; Chan et al., 1990; Janssens and Lesage, 2001) nor have been shown by us to be injured by oxygen deprivation times up to 12 hr (Fogal et al., 2005a), changes in LDH activity can be used as a specific marker of neuronal injury in this system.

2.3.9 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (Version 4.03, GraphPad Software, Inc.) as described in each figure legend. Percentage data were first transformed via arcsin square root. For qPCR, statistics were performed on the logarithmic retransformation (i.e. geometric means) of $2^{-\Delta\Delta CT}$ values. In all experiments, data are expressed as the mean ± SEM. Significance was assessed at p < 0.05.

2.4 Results

We previously demonstrated in a mixed cortical cell culture system that IL-1β potentiated neuronal injury induced by hypoxia (Fogal et al., 2005a) via a process dependent on increased system xc^- activity and impaired glutamate clearance (Fogal et al., 2007). Although, it was determined that this increase required astrocyte IL-1 receptor I (IL-1RI) signaling, the specific cell type(s) responding was not ascertained (Fogal et al., 2007). Hence, initial experiments
utilized purified cell culture preparations of neurons, astrocytes, and microglia to identify which cell types express xCT — the system $x_c^-$ light chain — and 4F2hc and RBAT — the system $x_c^-$ heavy chains — demonstrate functional cystine uptake, and respond to IL-1β by increasing system $x_c^-$ activity.

Under basal conditions, xCT, 4F2hc, and RBAT transcripts are present in mixed cortical cell cultures and in each of the purified populations of cells: astrocytes, neurons, and microglia (Figure 2.1). Further, all culture preparations functionally express system $x_c^-$ as determined by their ability to import radiolabeled cystine (Figure 2.2A-C; white bars). However, only astrocytes respond to IL-1β treatment (3 ng/ml; 20-24 hr) with an enhancement in cystine uptake (Figure 2.2A; black bars). No change in uptake was observed in pure neuronal or microglial cultures following IL-1β exposure at any time point assessed (Figure 2.2B,C).

Since IL-1β is known to regulate the expression of various genes, we next assessed whether its treatment (3 ng/ml) alters xCT steady-state mRNA expression in purified astrocyte, neuronal, and microglial cultures using quantitative PCR. Consistent with the uptake data, IL-1β elicits a time-dependent increase in xCT mRNA in astrocyte (Figure 2.3A), but not neuron or microglial cultures (Figure 2.3B), such that a 4-8 hr incubation produces $\approx$12-fold increase in astrocytic xCT mRNA (Figure 2.3A). Transcripts for the system $x_c^-$ heavy chains do not increase following IL-1β stimulation (Figure 2.3C).
The increase in astrocytic steady-state xCT mRNA that follows IL-1β treatment (3 ng/ml; 6 hr) is not observed in astrocytes derived from IL-1RI-/- mice (Figure 2.4A). Further, simultaneous exposure of pure astrocytes with IL-1β (3 ng/ml; 6 hr) and the transcriptional inhibitor actinomycin D (10 µg/ml; 6 hr) blocks the IL-1β-mediated increase in xCT mRNA expression; basal levels remain unchanged (Figure 2.4B). Consistent with the qPCR data, IL-1β treatment enhances protein levels of astrocyte xCT (Figure 2.5A,B), whereas 4F2hc levels are unaffected (Figure 2.5A,C). Finally, concomitant exposure of astrocytes to IL-1β (3 ng/ml) and actinomycin D (12.5 µg/ml; 24 hr), prevents the IL-1β-mediated enhancement in cystine uptake, as does incubation with the protein synthesis inhibitor cycloheximide (1 µg/ml; 24 hr) (Figure 2.6).
Figure 2.1. Cellular system $x_{c^+}$ expression. (A) Total RNA was isolated from unstimulated mixed cortical cell cultures (mix; lanes 1-2), pure astrocytes (lanes 3-5), pure neurons (lanes 6-8), and pure microglia (lanes 9-11), reverse transcribed, and PCR performed using specific primers for xCT (33 cycles), 4F2hc (33 cycles), RBAT (33 cycles) and β-actin (23 cycles) in separate reactions.
Figure 2.2. Astrocytes increase cystine uptake following IL-1β treatment. Astrocytes (A; n=4), neurons (B; n=4-8) and microglia (C; n=6-10) were treated with vehicle (white bars) or IL-1β (3 ng/ml; black bars) for 20-24 hr following which cells were washed and incubated with a buffer containing $^{14}$C-L-cystine (3 µM) and uptake was determined over time as indicated. Data are expressed as mean ± SEM $^{14}$C-L-cystine uptake in pmol/mg protein. An asterisk (*) indicates a significant between-group difference as determined by a two-way ANOVA followed by Bonferroni’s post hoc test. Significance was set at $p < 0.05$. 
Figure 2.3. IL-1β selectively increases astrocytic xCT mRNA. (A) Pure astrocytes (n=4) (B) neurons (n=4) and (inset) microglia (n=4) were treated with IL-1β (3ng/ml) or its vehicle for the indicated durations and xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to untreated cells (0 h) (C) Astrocytes (n=3) were treated with IL-1β (3ng/ml) or its vehicle for the indicated durations and 4F2hc and RBAT (inset) mRNA expression was assessed via qPCR. Data are expressed as mean ± SEM fold change in 4F2hc and RBAT mRNA compared to untreated cells (0 h). An asterisk (*) denotes values different from 0 hr as assessed by one-way ANOVA. Significance was set at p < 0.05.
Figure 2.4. IL-1R1 signaling and transcription are required for the enhancement in astrocyte xCT mRNA expression. (A) Astrocytes (n=4-5) were treated with IL-1β (3 ng/ml) or its vehicle for 6 h. Thereafter, total RNA was isolated, reverse transcribed and xCT and β-actin expression assessed using quantitative PCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to Il1r1 +/+ untreated cells (-IL-1β). (B) Astrocytes (n=5) were treated with IL-1β (3 ng/ml) or its vehicle in the presence and absence of actinomycin D (Act D; 10 µg/ml) and xCT mRNA expression assessed via qPCR 6 hr later. Data are expressed as mean ± SEM fold change in xCT mRNA compared to untreated cells (-IL-1β, -Act D). An asterisk (*) denotes values different from control and a pound sign (#) indicates values that significantly differ from IL-1β-treated conditions as assessed by two-way ANOVA followed by Bonferroni’s post hoc test. Significance was set at p <0.05.
**Figure 2.5. IL-1β increases xCT protein expression.** (A) Pure astrocyte cultures were incubated with vehicle or 3 ng/ml IL-1β in the absence or presence of cycloheximide (CHX 1 μg/ml) for 20-24 hr. Protein from unstimulated C6 glioma cells was used as a positive control for xCT protein. Lane 1, Basal; Lane 2, IL-1β; Lane 3, IL-1β+CHX, Lane 4, C6 positive control. Representative of two western blots. (B,C) Films were scanned and densitometry performed using Gelpro Analyzer software. (B) xCT and (C) 4F2hc protein levels were normalized to their corresponding β-actin protein levels and expressed as a fold increase (mean ± SEM; n = 2) over control (basal; set to 1).
We next set out to ascertain whether the astrocytic excitatory amino acid transporters (EAATs / system X\textsubscript{AG}\textsuperscript{−}) are coordinately regulated by IL-1\textbeta, since glutamate efflux via system x\textsubscript{c}\textsuperscript{−} is balanced by system X\textsubscript{AG}\textsuperscript{−}-mediated glutamate uptake (Danbolt, 2001; McBean, 2002). Treatment of astrocytes with IL-1\textbeta (3 ng/ml) does not enhance EAAT-1 (aka GLAST) or EAAT-2 (aka GLT-1) mRNA expression (Figure 2.7A) in the same time frame as it does xCT (Figure 2.3A). Additionally, there is no difference in astrocytic \textsuperscript{3}H-D-aspartate uptake—used here as a measure of EAAT activity—following a 24 hr exposure to IL-1\textbeta as compared to control (Figure 2.7B). Finally, the mRNA expression of LAT-2, a light chain of the System L amino acid transporter, is also unchanged by IL-1\textbeta stimulation (Figure 2.7A), further demonstrating target specificity of the IL-1\textbeta response. To elucidate the functional importance of these findings and definitively test whether astrocyte-mediated alterations in system x\textsubscript{c}\textsuperscript{−} activity contribute to the development and progression of inflammatory (IL-1\textbeta-enhanced) hypoxic neuronal injury, astrocytes derived from sut mice—which carry a functional mutation in xCT (Chintala et al., 2005)—were utilized. Since no PCR genotyping protocol currently exists to detect the truncated \textit{slc7a11} (\textit{sut}) gene, astrocytes were derived from the pooled cortices of the progeny obtained from heterozygous breeders (\textit{sut/±}).
Figure 2.6. Protein synthesis is required for the enhancement of astrocyte system $\nu^+_c$ activity that follows IL-1β treatment. Pure astrocyte cultures ($n = 11-12$) were treated with actinomycin D (ACT D; 12.5 µg/ml) or cycloheximide (CHX; 1 µg/ml) in the absence (white bars) or presence (black bars) of IL-1β (3 ng/ml) for 20-24 hr following which $^{14}$C-L-cystine uptake ($3 \mu$M labeled + $27\mu$M unlabeled; 25°C) was determined. Data are expressed as mean ± SEM $^{14}$C-L-cystine uptake in pmol/30 min/mg protein. An asterisk (*) denotes values different from control (-IL-1β) and a pound sign (#) indicates values different from IL-1β-treated conditions as assessed by two-way ANOVA followed by Bonferroni’s post hoc test. Significance was set at $p < 0.05$. 
Figure 2.7. IL-1β does not regulate mRNA expression or activity of system X_{AG} amino acid transporters. (A) Pure astrocyte cultures (n = 3-4) were treated with IL-1β (3ng/ml) or its vehicle for the indicated durations and EAAT-1, EAAT-2, and LAT2 mRNA expression assessed via qPCR. Data are expressed as mean ± SEM fold change in mRNA compared to untreated cells (0 h). (B) Pure astrocyte cultures (n =10) were treated with vehicle (white bars) or IL-1β (3 ng/ml; black bars) for 20-24 hr following which ³H-D-aspartate (0.1 µCi/ml labeled + 1-100 µM unlabeled; 25°C) uptake was determined. Data are expressed as mean ± SEM ³H-D-aspartate uptake in cpm x 10³/5 min/mg protein. No significant between-group differences were found via two-way ANOVA.
According to Mendelian inheritance (¼+/+, ½ sut/+, ¼ sut/sut), cultures should have ≈50% reduction in functional sut gene expression. Indeed, astrocytes derived from sut animals demonstrate 61 ± 6% less cystine uptake compared to WT astrocytes when cultured under basal conditions (Figure 2.8A). Moreover, sut astrocytes do not respond to IL-1β with an increase in cystine uptake as do cultures derived from wild-type controls (Figure 2.8A). Finally, the enhanced neuronal vulnerability to hypoxia that follows IL-1β treatment in our mixed culture system (Fogal et al., 2007) –which is recapitulated herein (Figure 2.8B, 2.9)– is not observed in chimeric cultures consisting of wild-type neurons plated on top of sut astrocytes (Figure 2.8B, Appendix Figure A2). Nor is it observed in wild-type cultures treated with a system xc⁻ inhibitor (50 μM LY367385) or an NMDA receptor antagonist (10 μM MK-801) (Figure 2.9), as was similarly demonstrated by us previously (Fogal et al., 2007). Together, these data demonstrate that IL-1β selectively regulates system xc⁻ activity in astrocytes and that this change is specifically responsible for the deleterious, excitotoxic effects of IL-1β found under hypoxic conditions.
Figure 2.8. Cystine uptake and hypoxic neuronal cell death are reduced in cultures containing *sut* astrocytes. (A) Pure astrocyte cultures (n = 5-6) derived from either wild-type (white bars) or *sut* mice (black bars) [all cultured w/ 55 µM β-ME] were treated with vehicle or IL-1β (3 ng/ml) for 20-24 hr after which 14C-L-cystine uptake was determined. Data are expressed as mean ± SEM 14C-L-cystine uptake in pmol/30 min/mg protein. (B) Chimeric mixed cortical cell cultures were obtained by plating WT neurons on astrocytes derived from *sut* mice (black bars). These and control cultures (WT neurons on WT astrocytes; white bars) were treated with 1 ng/ml IL-1β or vehicle for 20-24 hr, washed, and then deprived of oxygen for 5 hr. The percentage of total neuronal cell death was determined 20-24 hr later (n = 4 cultures pooled from two independent experiments). An asterisk (*) indicates a significant within-group difference, while a pound (#) sign indicates a significant between-group difference as determined by a two-way ANOVA followed by Bonferroni’s post hoc test. Significance was set at p < 0.05.
Figure 2.9. Ionotropic glutamate receptor and system x_c^- antagonism prevent IL-1β-mediated hypoxic neuronal injury. Mixed cortical cell cultures were treated with 3 ng/ml IL-1β for 20-24 hr, washed, and then deprived of oxygen for 4 hr. The ionotropic glutamate receptor antagonist MK-801 (10 µM) and the system x_c^- antagonist LY367385 (50 µM) were added at the initiation of hypoxia. The percentage of total neuronal cell death was determined 20-24 hr later (n = 5-6 cultures pooled from 2 independent experiments). An asterisk (*) denotes values different from control untreated cultures (hypoxia) and a pound sign (#) indicates values different from IL-1β-treated conditions as assessed by one-way ANOVA followed by a Student-Newman-Keul’s post hoc test. Significance was set at p < 0.05.
2.5 Discussion

Several studies have demonstrated that system \( x_c \) is an important contributor to the ambient extracellular glutamate levels that bathe the central nervous system (Jabaudon et al., 1999; Warr et al., 1999; Baker et al., 2002a; Baker et al., 2002c; Melendez et al., 2005; Augustin et al., 2007; Featherstone and Shippy, 2008). Additionally, system \( x_c \) activity has been demonstrated to control synapse strength and courtship behavior in drosophila (Grosjean et al., 2008), as well as, drug seeking and sensitization behavior in rodents (Baker et al., 2002a; Moran et al., 2005; Baker et al., 2008). Finally, its activity can also contribute to neuropathology. For instance, export of glutamate via system \( x_c \) produces an excitotoxic necrosis that aids in glioma tumor growth, migration, and invasion (Ye et al., 1999; Ye and Sontheimer, 1999; Lyons et al., 2007; Savaskan et al., 2008; Sontheimer, 2008). Further, the deleterious effect of A\( \beta \)-, secreted \( \beta \)-amyloid precursor protein (sAPP)- or LPS-treated microglia or IL-1\( \beta \)-treated mixed cultures on neuronal and oligodendrocyte survival in vitro has been shown to be caused by system \( x_c \)-mediated excitotoxicity (Piani and Fontana, 1994; Barger and Basile, 2001; Qin et al., 2006; Domercq et al., 2007; Fogal et al., 2007). Thus, understanding the regulation of system \( x_c \) at the cellular and molecular level is of great import. Toward this end, the central observation of this study is that IL-1\( \beta \) enhances the functional expression of system \( x_c \) in astrocytes specifically and selectively via a process dependent on IL-1R1 signaling and de novo protein synthesis. Of pathological relevance, this IL-1\( \beta \)-mediated increase in astrocytic system \( x_c \) activity enhances neuronal injury initiated by hypoxia.
Demonstration of the expression of xCT, 4F2hc and RBAT mRNA in purified populations of neurons, astrocytes and microglia indicate that each cell type has the molecular machinery necessary for the formation of a functional system $x_c^{-}$ antiporter (Figure 2.1). That these transcripts are translated to functional protein is demonstrated by the ability of each cell type to take up cystine in a system $x_c^{-}$-dependent manner (Figure 2.2). While others have demonstrated xCT mRNA expression in neurons (Dun et al., 2006; Ogawa et al., 2008) and retinal Muller cells (Tomi et al., 2003; Mysona et al., 2009), this is the first report of xCT mRNA expression in astrocytes and microglia, although xCT protein expression has been demonstrated in all three cell types (Burdo et al., 2006; Dun et al., 2006; Domercq et al., 2007; La Bella et al., 2007). Previous studies have also demonstrated functional system $x_c^{-}$ activity in neurons (Murphy et al., 1990; Dun et al., 2006), astrocytes (Cho and Bannai, 1990; Bender et al., 2000; Pow, 2001; Tang and Kalivas, 2003; Lewerenz et al., 2009a), and microglia (Piani and Fontana, 1994; Barger and Basile, 2001; Nakamura et al., 2003; Barger et al., 2007; Domercq et al., 2007).

Interestingly, of the three cell types studied, only astrocytes respond to IL-1β by increasing the mRNA of the system $x_c^{-}$ light chain xCT – though not the heavy chains (Figure 2.3A,C) – and by increasing cystine uptake (Figure 2.2A). Additionally, IL-1β induces expression of xCT protein in astrocytes (Figure 2.5). As increases in mRNA do not always translate to similar changes in protein expression, it is not surprising that $\approx 12$-fold increase in xCT mRNA expression (Figures 2.3, 2.4) only resulted in an approximate four-fold change in xCT protein
expression (Figure 2.5). Additionally, the discrepancy between changes in protein levels (4 fold) and the increase in cystine uptake (2 fold) (Figures 2.6) may occur as a function of the experimental system utilized to specifically isolate cystine transport via system $x_c^-$. The presence of 1mM D-aspartate –used to inhibit $X_{AG}^-$-mediated cystine uptake – is known to alter the driving force required for optimal system $x_c^-$ activity (Reichelt et al., 1997).

Conclusive demonstration that the IL-1β-mediated enhancement in cystine uptake is mediated by astrocytic system $x_c^-$ comes from our observation that astrocytes derived from sut animals (Swank et al., 1996; Chintala et al., 2005) fail to demonstrate this effect (Figure 2.8A). Both of these results are consistent with several studies, in neural and non-neural systems, which demonstrate an association between xCT mRNA expression and system $x_c^-$ activity (Bridges et al., 2001; Sato et al., 2001; Tomi et al., 2003; Sato et al., 2004; Dun et al., 2006; Mysona et al., 2009). The lack of coordinate regulation of the subunits might not be too surprising as the heavy chains are utilized by other transport systems and as such exist in cells in excess (Stevens and Vo, 1998; Verrey et al., 2004). It should be noted, however, that there is at least one study that describes a parallel increase in xCT and 4F2hc mRNA occurring in response to LPS (Sato et al., 2001).

The fact that microglia system $x_c^-$ components (Figure 2.3B) and activity (Figure 2.2C) are unaffected by treatment with IL-1β is consistent with other studies utilizing cells of the macrophage/monocyte lineage (Piani and Fontana, 1994; Sato et al., 1995a). This finding may be due to the fact that microglia have
a low ratio of signaling (i.e. IL-1RI) to decoy (i.e. IL-1RII) receptors making them relatively unresponsive to IL-1β (Pinteaux et al., 2002). Whether this same mechanism accounts for the inability of IL-1β to alter system x_c^- in neurons remains to be determined. Additionally, it is possible that the differential signaling that follows IL-1RI activation in neurons and astrocytes (Srinivasan et al., 2004) fosters xCT regulation in one cell type and not the other.

The IL-1β-mediated increase in steady-state xCT mRNA is due, at least in part, to the initiation of transcription following IL-1RI activation (Figure 2.4). Whether the latter occurs via activation of transcription factors known to facilitate transcription of the xCT promoter in response to amino acid deprivation, LPS, and oxidative stress (e.g., Nrf2 and ATF4) (Sasaki et al., 2002; Sato et al., 2004; Lewerenz et al., 2009a) remains to be determined. The failure of IL-1β to increase xCT protein expression (Figure 2.5) and cystine uptake (Figure 2.6) in the presence of cycloheximide suggests that de novo synthesis and subsequent insertion of a functional transporter into the membrane are required. Cycloheximide also prevented the enhancement of transporter activity mediated by LPS in mouse microglia (Piani and Fontana, 1994), by glucose/glucose oxidase (i.e. oxidative stress) in human endothelial cells (Miura et al., 1992), and by an NO donor in retinal pigment epithelial cells (Bridges et al., 2001). Nevertheless, the requirement of transcription and translation may be cell, species, and/or stimulus-specific (Barger et al., 2007). Additionally, post-transcriptional regulation of system x_c^- components and activity in astrocytes in response to the antibiotic ceftriaxone has recently been described (Lewerenz et
Not only does IL-1β demonstrate cellular specificity, but it appears to show target specificity as well. IL-1β had no effect on the expression of the system L transporter light chain, LAT2, or the expression and function of astrocytic excitatory amino acid transporters, EAAT-1 and EAAT-2 (Figure 2.7). System L, and LAT-2 in particular, was chosen as a potential target as it is expressed in brain (Segawa et al., 1999), it mediates the uptake of the neutral amino acids cysteine and methionine (Oxender et al., 1977; Sato et al., 1987; Segawa et al., 1999), and because methionine can be converted in the brain to cysteine via transulfuration, a process linked to GSH biosynthesis/homeostasis (Vitvitsky et al., 2006). EAATs were assessed as it is possible that glutamate import machinery could be cooperatively regulated to maintain low extracellular glutamate levels. The lack of effect on the EAATs may not be surprising considering the abundance at which they are expressed in astrocytes (Bergles and Jahr, 1997; Lehre and Danbolt, 1998). Additionally, this agrees with previous studies that note no alterations in system X_{AG}^− function in the face of increased system x_c^− activity (Lewerenz et al., 2009a; Mysona et al., 2009).

The data described herein coupled with our previous studies (Fogal et al., 2005a; Fogal et al., 2007) suggest the following scenario (Figure 2.10). As System x_c^− is an obligate exchanger, the import of cystine is coupled to glutamate export. Under physiological conditions, the accumulation of glutamate is prevented by its rapid clearance from the extracellular space via system X_{AG}^−. Consequently, no neuronal toxicity is observed — demonstrating that increased
activity of system $x_c$ is not inherently injurious (Fogal et al., 2005a). In contrast, when glutamate uptake is impaired, increased system $x_c$ activity can result in the accumulation of extracellular glutamate and subsequent excitotoxic neuronal cell death (Fogal et al., 2007). This work advances our previous study by demonstrating unequivocally that IL-1$\beta$ enhances system $x_c$ activity in astrocytes exclusively (Figure 2.2) and that this increase is responsible for the potentiation of neuronal injury found under hypoxic conditions. To wit, sut astrocytes — with impaired functional system $x_c$ — neither increase cystine uptake in response to IL-1$\beta$ (Figure 2.8A) nor support the ability of IL-1$\beta$ to mediate hypoxic neuronal injury when co-cultured with neurons (Figure 2.8B).

In summary, we have analyzed expression of xCT mRNA and the activity of system $x_c$ in purified astrocyte, neuron, and microglial cultures in the presence and absence of IL-1$\beta$, a cytokine known to be upregulated in and to contribute to various neurological disorders [for review see (Fogal and Hewett, 2008)]. The results unequivocally demonstrate that astrocytes increase system $x_c$ expression and activity in response to IL-1$\beta$, whereas neurons and microglia do not. The enhancement in astrocytic transporter activity requires transcription and translation, demonstrates specificity for the xCT subunit, and is responsible for the increase in hypoxic inflammatory (IL-1$\beta$-mediated) neuronal cell death.
Figure 2.10. Schematic of the system $x_c^-$ amino acid transporter under normoxic and hypoxic conditions. The import of cystine via system $x_c^-$ is directly coupled to glutamate export, occurring in a Na$^+$-independent, Cl$^-$-dependent manner with 1:1 stoichiometry. $X_{AG}^-$ transports glutamate into the cell to maintain the driving force for system $x_c^-$ (Reichelt et al., 1997) (left panel). Under hypoxic conditions, enhanced export of glutamate via system $x_c^-$ in combination with reduced glutamate clearance by system $X_{AG}^-$ produces an accumulation of extracellular glutamate which contributes to excitotoxic neuronal cell death (right panel). [Based on results described herein as well as those found in Fogal et al., 2005, 2007].
CHAPTER 3

Non-cell autonomous influence of astrocyte system $x_c^*$ on hypoglycemic neuronal cell death
3.1 Summary

Despite longstanding evidence that hypoglycemic neuronal injury is mediated by glutamate excitotoxicity, the cellular and molecular mechanisms involved remain incompletely defined. Here, we demonstrate that the excitotoxic neuronal injury that follows glucose deprivation is mediated by glutamate extruded from astrocytes via system xᵅ⁻ an amino acid transporter that imports L-cystine and exports L-glutamate. Specifically, we find that depriving mixed cortical cell cultures of glucose for up to 8 hr results in increased extracellular glutamate \([\text{glutamate}]_e\) levels that injure neurons, but not astrocytes. Neuronal injury is prevented by ionotropic glutamate receptor antagonism but is insensitive to tetanus toxin. Removal of amino acids during the deprivation period prevents — whereas addition of L-cystine restores — the enhancement of \([\text{glutamate}]_e\) and neuronal injury, implicating the cystine/glutamate antiporter, system xᵅ⁻. Drugs known to inhibit system xᵅ⁻ also suppress glucose deprivation-induced enhancement of \([\text{glutamate}]_e\) and neuronal injury. Further, a dramatic reduction in neuronal injury is observed in chimeric cultures consisting of neurons derived from wild-type mice plated on top of astrocytes derived from su:t mice, which harbors a naturally occurring null mutation in the gene (\text{Slc7a11}) which encodes the substrate specific light chain of system xᵅ⁻ (xCT). Finally, enhancement of astrocytic system xᵅ⁻ expression and function via exposure to IL-1β potentiates hypoglycemic neuronal injury, the process of which is prevented by removal of L-cystine and/or addition of system xᵅ⁻ inhibitors. Thus — under the conditions of
glucose deprivation — our studies demonstrate that astrocytes, via system $x_c^-$, have a direct, non–cell autonomous effect on cortical neuron survival.

3.2 Introduction

Hypoglycemia is a medical emergency that arises as a serious complication of insulin therapy in diabetic patients. It is also prevalent in neonates, in patients with insulin-producing tumors, and can occur as a consequence of brain ischemia. Severe hypoglycemia, defined as less than 2 mM blood glucose, essentially renders the brain aglycemic, leading to cognitive impairments and frank neuronal injury (Ryan et al., 1990; Langan et al., 1991). Evidence indicates that hypoglycemic/aglycemic neuronal cell death is mediated by glutamate excitotoxicity (Wieloch, 1985; Monyer et al., 1989). Following activation of glutamate receptors, a cascade of biochemical events is initiated that ultimately leads to neuronal cell death via processes dependent on reactive oxygen species, neuronal zinc release, activation of poly(ADP-Ribose) polymerase-1 (PARP), and alterations in mitochondrial function. Inhibition of these downstream targets of glutamate receptor activation show some success in reducing hypoglycemic brain injury [for review see (Suh et al., 2007)]. However, the cellular and molecular mechanisms surrounding glutamate release/accumulation remain incompletely defined.

System $x_c^-$ is a heteromeric amino acid transporter consisting of two subunits: xCT — the light chain conferring substrate specificity — and a heavy
chain thought to target the transporter to the plasma membrane (Sato et al., 1999; Bassi et al., 2001). The import of cystine via system \( x_{\text{c}}^- \) is directly coupled to glutamate export, which occurs in a \( \text{Na}^+ \)-independent manner (Bannai and Kitamura, 1980). Enhanced system \( x_{\text{c}}^- \) activity has been previously reported to contribute to excitotoxic neuronal injury in numerous experimental paradigms (Piani and Fontana, 1994; Ye et al., 1999; Barger and Basile, 2001; Qin et al., 2006; Fogal et al., 2007; Savaskan et al., 2008; Sontheimer, 2008; Jackman et al., 2010; Massie et al., 2010a). Herein, we describe astrocytic system \( x_{\text{c}}^- \) as the source of glutamate required for the initiation of non-cell autonomous neuronal injury following glucose deprivation \textit{in vitro}.

### 3.3 Materials and Methods

#### 3.3.1 Cell culture

All media including media stock (MS), glial plating medium, mixed culture plating medium, maintenance medium, and glucose-free balanced salt solution (BSS) have been fully described (see Chapter 2.3.1). For most experiments, BSS\(_0\) contains purchased 1x MEM amino acids (Invitrogen). Amino acids were reconstituted individually for the removal and addition experiments.

Primary astrocyte cultures were derived from cerebral cortices of day 1-3 postnatal CD1 mouse pups (Charles River) as described (Trackey et al., 2001; Jackman et al., 2010). Wild-type (WT) and sut (xCT-deficient) astrocytes were cultured from cortices of single pups derived from \( sut/+ \) breeding pairs (JAX; Stock # 001310). \( \beta \)-mercaptoethanol (\( \beta \)-ME; 55 \( \mu \)M) was added to the glial
plating medium of *sut* cultures to support growth and to WT cultures for control purposes (Shih et al., 2006). The rest of the brain was used for genotyping: WT primers (230 bp product) 5'- GAA GTG CTC CGT GAA GAA GG 3' (forward), 5'-ATC TCA ATC CTG GGC AGA TG-3' (reverse); *sut* primers (≈2280 bp product) 5'-CCA CTG TTG TAG GTC AGC TTA GG-3' (forward), 5'-CAG GAC CTG TGA ATA TGA TAG GG 3' (reverse).

*Mixed cortical cell cultures* containing predominantly neurons and astrocytes were prepared by culturing dissociated cells from embryonic day 15 mouse fetuses onto a confluent layer of astrocytes in mixed culture plating media as previously described (Trackey et al., 2001; Jackman et al., 2010). Experiments were performed on mixed cortical cultures after 13-14 days *in vitro*.

### 3.3.2 Glucose Deprivation

Mixed cortical cell cultures were washed thoroughly (8 x 750 µL) into BSS$_0$. Glucose (final concentration=10mM) was immediately added to parallel cultures to serve as controls and added back to experimental conditions to terminate glucose deprivation at the times indicated in each figure legend. Inhibitors — with the exception of an overnight incubation with tetanus toxin — were given at the initiation of glucose deprivation.

### 3.3.3 Glutamate Uptake

System $X_{AG}^-$-mediated $^3$H-D-aspartate (PerkinElmer) uptake was performed as previously described (Fogal et al., 2007; see Chapter 2.3.3) using
0.1 μCi/ml $^3$H-D-aspartate and 50 μM unlabeled D-aspartate (5 min; 25°C). Accumulated radioactivity was estimated using a liquid scintillation counter and values normalized to protein [BCA Assay (Pierce)].

3.3.4 Measurement of cell death

Cell death was quantitatively determined by spectrophotometric measurement of lactate dehydrogenase (LDH) as described previously (Uliasz and Hewett, 2000). Data are expressed as a percentage of total neuronal LDH activity (defined as 100%) determined by exposing parallel cultures to 250 μM NMDA (20-24 hr). Since primary cortical astrocytes neither express NMDA receptors nor are injured by glucose deprivation up to 8 hr (Figure 3.1 inset), changes in LDH activity can be used as a specific marker of neuronal injury. In general, basal LDH released from control cultures was subtracted from values obtained in experimental conditions to yield the signal specific to hypoglycemic injury. Astrocyte injury was quantified as a percentage of total astrocytic LDH activity (defined as 100%) determined by exposure of parallel cultures to 5 μM Calphostin C for 20-24 hr (Ikemoto et al., 1995).

3.3.5 Measurement of glutamate

Samples of the bathing media from the mixed cortical cultures were stored at -80°C until analyzed. Two hundred microliters were assayed for glutamate via HPLC by an investigator blinded to the treatment conditions as described (Fogal
et al., 2007). Glutamate levels were calculated by normalizing to glutamate standards which were linear over the range of 0.1–100 µM.

3.3.6 IL-1β Treatment

To selectively enhance astrocytic system \( x_c^- \) expression/activity (Jackman et al., 2010), cultures were treated with 0.01-1 ng/ml recombinant murine IL-1β (R&D Systems) in an incubation buffer of MS supplemented with 0.1% fatty-acid free BSA (Sigma).

3.3.7 Quantitative Real-time PCR (qPCR)

Quantitative PCR was performed using mouse-specific primer pairs [Taqman Gene Expression Assays, Applied Biosystems: \( x_C \) (Mm00442530_m1)] per manufacturer’s instructions using the comparative cycle threshold method (\( \Delta\Delta C_T \)) with \( \beta \)-actin as the housekeeping control as described (Jackman et al., 2010). \( \beta \)-actin \( C_T \) values were unaffected by IL-1β treatment. Amplification efficiency was >94%.

3.3.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism Software (Version 4.03) as described in each figure legend. Percentage data were first transformed via arcsin square root. For qPCR, statistics were performed on the logarithmic retransformation (i.e. geometric means) of \( 2^{\Delta\Delta C_T} \) values. In all
experiments, data are expressed as the mean ± SEM. Significance was assessed at \( p < 0.05 \).

### 3.4 Results

Following glucose deprivation, there is a time-dependent increase in neuronal injury in the mixed cortical cultures (Figure 3.1A), whereas purified astrocytes (Figure 3.1 inset) and astrocytes in mixed cultures are resistant for up to 8 hr, the longest time-point assessed. Neuronal injury is associated with accumulation of glutamate in the tissue culture supernatant (Figure 3.1B) and is prevented by ionotrophic glutamate receptor antagonism (Figure 3.1C). Overnight incubation with 300 ng/ml tetanus toxin — a concentration which cleaves neuronal synaptobrevin-2 (data not shown) and blocks depolarization-induced glutamate release in our system (Taylor and Hewett, 2002; Fogal et al., 2005b) — failed to attenuate neuronal damage (Figure 3.1D). Alterations in classical glutamate re-uptake transporter function also did not appear to contribute to the observed accumulation of \([\text{glutamate}]_e\) as \(^3\text{H}-\text{D-aspartate}\) uptake under control conditions and following 6 h of glucose deprivation in astrocytes (Figure 3.2A) and in mixed cultures (Figure 3.2B) did not differ statistically. Notably, in mixed cultures with concentrations of cold D-aspartate that span the \(K_m\)'s of all glutamate transporters, no difference in uptake was observed under hypoglycemic conditions (Figure 3.2B). Further, glucose deprivation did not alter system \( x_c^- \) activity as assessed via the uptake of radiolabeled cystine (Figure 3.3).
Both neuronal injury and glutamate accumulation resulting from glucose deprivation was, however, inhibited by 4-CPG or LY367385 (each at 50 µM) but not YM298198 (10 µM) (Figure 3.4). The former are drugs that inhibit both system $x_c^-$ and mGluR1α, whereas the latter is a selective mGluR1α antagonist (Fogal et al., 2007). Complete removal of amino acids during the deprivation period prevented — whereas addition of L-cystine alone restored — the enhanced $[\text{glutamate}]_e$ and hypoglycemic neuronal injury, further supporting a role for system $x_c^-$ (Figure 3.5A,C). Likewise, the solitary removal of L-cystine during the glucose deprivation period prevented hypoglycemic neuronal cell death. Removal and/or addition of L-methionine had no effect (Figure 3.5 A-C), attesting to the requirement of L-cystine.
Figure 3.1. Non-synaptically released glutamate contributes to hypoglycemic neuronal injury. Mixed cortical cultures or astrocytes (inset) were incubated in media containing (hatched bars) or lacking (black bars) glucose. The percentage of total cell death (A) and glutamate accumulation in the tissue culture supernatant (B) were determined at the times indicated. Between group differences (*) were determined by one-way (astrocytes) or two-way ANOVA (mixed cultures) followed by Bonferroni’s post hoc test (n=11-12 cultures from four different experiments). (C) Mixed cortical cultures were washed into BSS0 containing vehicle, MK-801 (10 µM), or MK-801 plus CNQX (30 µM) for 8 hr (GD). Neuronal injury was determined 20-24 hr later. (*) indicates values significantly different from control conditions (10.56 ± 3.02%); (#) represents a significant diminution of GD-induced injury [one-way ANOVA followed by Student-Newman-Keul’s post hoc test]. (n=11 cultures from three separate experiments). (D) Mixed cultures were incubated overnight with vehicle or tetanus toxin (TeNT; 300 ng/ml), then washed into BSS0. Eight hr later, glucose (10 mM) was added and the cultures were placed back into the incubator, after which neuronal injury was assessed (20-24 hr). (*) indicates values significantly different from control conditions (5.71 ± 2.03%) [one-way ANOVA followed by Student-Newman-Keul’s post hoc test]. No between-group differences exist (n = 8-9 cultures from three separate experiments).
Figure 3.2 Hypoglycemia does not impair glutamate uptake in astrocytes or mixed cultures. (A) Pure astrocytes (n=10 culture wells from 2 independent experiments) were incubated in media containing (hatched bars) or lacking (black bars) glucose for 1-6 hr after which $^3$H-D-aspartate (0.1 μCi/ml labeled + 50 μM unlabeled; 25°C) uptake was determined in the presence and absence of the competitive, non-transportable EAAT inhibitor TBOA (500 μM). (B) Mixed cortical cultures (n =6 from 2 independent experiments) were incubated in media containing (hatched bars) or lacking (black bars) glucose for 6 hr after which $^3$H-D-aspartate (0.1 μCi/ml labeled + 1-100 μM unlabeled; 25°C) uptake was determined. Data are expressed as mean ± SEM $^3$H-D-aspartate uptake in cpm /5 min/mg protein. No significant between-group differences were found via two-way ANOVA.
Figure 3.3. Hypoglycemia does not increase the activity of system $x_c^-$. Mixed cortical cultures ($n = 6$) were deprived of glucose for 0-8 hr after which they were incubated in a buffer containing $^{14}$C-L-cystine (3 µM; 25°C) and uptake was determined over 0 minutes. Data are expressed as mean ± SEM $^{14}$C-L-cystine uptake in pmol/mg protein. No significant differences were found via one-way ANOVA.
Figure 3.4. System $x_c^-$ antagonism prevents glutamate accumulation and neuronal cell death. (A,B) Mixed cortical cultures were washed into BSS in the presence or absence of 4-CPG (50 µM), LY367385 (50 µM), or YM298198 (10 µM). Eight hr later, supernatant was collected for measurement of neuronal cell death (A) and glutamate accumulation (B). (*) indicates values that are significantly different from control conditions (7.98 ± 0.89 % neuronal injury; 9.56 ± 2.29 µM glutamate), while (#) represents a significant diminution from GD-induced (A) neuronal injury or (B) glutamate accumulation [one-way ANOVA followed by Student-Newman-Keul's post hoc test. (n=11-12 cultures from three different experiments)]. (Right) Representative phase micrographs of mixed cortical cultures: (a) control; (b) 8 h GD; (c) GD + 4-CPG (50 µM); (d) GD + YM298198 (10 µM).
To determine whether cell-autonomous/non-cell autonomous alterations in system $x_c^-$ activity underlie the initiation and/or progression of neuronal injury following glucose deprivation, we prepared chimeric cultures consisting of WT neurons plated on top of astrocytes derived from mice harboring a null mutation in \textit{Slc7a11} (\textit{sut} gene), which encodes for xCT, the light subunit of system $x_c^-$ (Chintala et al., 2005). In comparison to cultures containing both WT neurons and astrocytes, neuronal injury following glucose deprivation is dramatically reduced in chimeric cultures consisting of wild-type neurons plated on top of \textit{sut} astrocytes (Figure 3.6). Notably, the comparable LDH levels measured following an NMDA exposure that results in 100\% neuronal cell death (Figure 3.6 inset) demonstrate that neurons plated on wild-type or \textit{sut} astrocytes have similar growth properties / cell densities and that wild-type neurons plated on \textit{sut} astrocytes are sensitive to NMDA. Finally, treatment of mixed cultures with IL-1β to enhance xCT expression (Figure 3.7A) and system $x_c^-$ activity in astrocytes (Jackman et al., 2010a) — potentiates hypoglycemic neuronal injury (Figure 3.7B) such that injury now occurs at an earlier time point. This enhanced neuronal injury is blocked by system $x_c^-$ antagonism and/or by removal of the substrate, L-cystine (Figure 3.7C).
Figure 3.5. Hypoglycemic neuronal injury is dependent on cystine. Cultures were deprived of glucose (4 hr) in a medium containing (GD) or lacking (-AA) MEM amino acids save for supplementation with L-cystine (+CYSS) or L-methionine (+MET). (*) indicates values different from control (2.87 ± 0.38 % injury; 5.7 ± 0.74 µM glutamate) while (#) represents a significant diminution from GD-induced (A) neuronal injury or (B) glutamate accumulation [one-way ANOVA followed by Student-Newman-Keul’s post hoc test. (n = 4)]. (C) Cultures were deprived of glucose (4 hr) in a medium containing (GD) or lacking (-AA) MEM amino acids, L-cystine (-CYSS) alone, or L-methionine (-MET) alone. (*) indicates values significantly different from control (0.55 ± 0.28 % injury); (#) represents a significant diminution from GD-induced neuronal injury [one-way ANOVA followed by Student-Newman-Keul’s post hoc test. (n=24 cultures from six separate experiments)].
Figure 3.6. Hypoglycemic neuronal injury is dependent on astrocyte system x_{c}. Chimeric cultures were obtained by plating WT neurons on astrocytes derived from *sut* mice (hatched white bars). These and control cultures (WT neurons on WT astrocytes; black bars) were washed into BSS_{0} (8 hr), glucose added (10 mM), and then neuronal cell death determined 20-24 hr later. (*) indicates a significant within-group difference, while (#) indicates a significant between-group difference [two-way ANOVA followed by Bonferroni’s post hoc test. (n = 15-18 cultures from three separate experiments)]. *Inset:* LDH absorbance values for chimeric and control cultures treated with 250 µM NMDA for 20-24 hr.
Figure 3.7. Enhanced system x\textsubscript{c}\textsuperscript{-} activity potentiates hypoglycemic neuronal injury. (A) Pure astrocytes (n = 3 from three experiments) were incubated with IL-1\textbeta or vehicle for 6 hr, after which xCT mRNA expression was assessed. (*) denotes values different from 0 hr [one-way ANOVA followed by Dunnett’s post hoc test]. (B) Mixed cultures were incubated with IL-1\textbeta for 20-24 hr then washed into BSS\textsubscript{0}. Glucose was added after 3.5 hr and neuronal cell death determined 20-24 hr later. (*) indicates values different from control (0 ng/ml IL-1\textbeta) [one-way ANOVA followed by Dunnett’s post hoc test (n = 16 cultures from four separate experiments)]. (C) Mixed cultures were incubated with IL-1\textbeta (GD + IL-1\textbeta) or vehicle (GD) for 20-24 hr, then washed into BSS\textsubscript{0} containing 4-CPG (50 \muM) or LY367385 (50 \muM) or one lacking cystine (-CYSS) for 4 hr. (*) indicates values different from GD. (#) represents a significant diminution from the IL-1\textbeta-mediated potentiation of GD-induced neuronal injury [one-way ANOVA followed by Student-Newman-Keul’s post hoc test (n=6-16 cultures from four separate experiments)].
3.5 Discussion

When blood glucose concentrations fall below 2 mM (normal = 4-7 mM), brain glucose levels approach zero (Silver and Erecinska, 1994; Choi et al., 2001), precipitating neuronal injury. Neurons are highly sensitive to glucose deprivation (Auer et al., 1984), whereas astrocytes have been demonstrated to be more resistant (Monyer et al., 1989; Lyons and Kettenmann, 1998). This is in accordance with the findings that rapid ATP depletion occurs exclusively in neurons following glucose deprivation in vitro (Choi et al., 2008) and that astrocytes contain glycogen stores that can be rapidly mobilized to meet metabolic needs during conditions of glucose deprivation (Cataldo and Broadwell, 1986; Swanson and Choi, 1993).

Despite this, cell death does not appear to be a direct result of energy failure. In fact, several studies demonstrate that hypoglycemic neuronal injury occurs secondary to glutamate excitotoxicity, as insulin-induced hypoglycemia results in a two-three fold increase in glutamate accumulation in the rat hippocampus and striatum as measured by microdialysis (Sandberg et al., 1986; Silverstein et al., 1990). The data presented herein are in agreement with a two-fold change in extracellular glutamate. Further, ionotropic glutamate receptor antagonists are protective both in vivo and in vitro (Wieloch, 1985; Monyer et al., 1989). Nevertheless, questions concerning the cellular source and molecular mechanisms surrounding glutamate release/accumulation following hypoglycemia remain.
Although, *in vivo* deafferentation studies suggest some neuronal contribution to hypoglycemic neuronal injury (Wieloch et al., 1985), we find that synaptic release of glutamate did not contribute to toxicity, as glucose-deprivation-induced neuronal injury was not prevented by incubation with tetanus toxin at a concentration that cleaves neuronal synaptobrevin-2 and blocks depolarization-induced glutamate release/injury in our system (Taylor and Hewett, 2002; Fogal et al., 2005b). This contrasts with earlier work demonstrating 40% protection against glucose deprivation-induced neuronal injury following tetanus toxin exposure, albeit at a concentration 10x higher (Monyer et al., 1992). Even when tested at the highest concentration utilized by Monyer and colleagues, there was only 15% protection (data not shown), suggesting that in our system synaptically-released glutamate is a minor source of excitotoxic levels of glutamate. Additionally, we did not observe a reduction in glutamate (\(^{3}\text{H}-\text{D-aspartate}\)) uptake following 6 h of glucose deprivation. This agrees with literature demonstrating no change in glutamate uptake in primary mouse cortical astrocytes following 2 h of glucose deprivation (Bakken et al., 1998) and only a 20% loss after 24 h (Swanson and Benington, 1996). This begs the question as to the cellular source of glutamate.

Studies have demonstrated that system \(x_{c}\), the antiporter that extrudes glutamate while importing cystine, is an important contributor to the ambient extracellular glutamate levels that bathe the central nervous system (Warr et al., 1999; Baker et al., 2002b; Augustin et al., 2007; Massie et al., 2010b; De Bundel et al., 2011). Importantly, the activity of system \(x_{c}\) has also been linked to
excitotoxic neuronal injury in a number of paradigms (Piani and Fontana, 1994; Barger and Basile, 2001; Qin et al., 2006; Fogal et al., 2007). For instance, the export of glutamate via system $x_c$- produces an excitotoxic necrosis that aids in glioma tumor growth and invasion (Savaskan et al., 2008; Sontheimer, 2008) and IL-1β-mediated hypoxic neuronal injury and 6-hydroxydopamine-mediated dopaminergic toxicity are dependent on system $x_c$- (Fogal et al., 2007; Jackman et al., 2010; Massie et al., 2010a). We now report that hypoglycemic neuronal toxicity is cystine-dependent and abrogated by system $x_c$- antagonism (Figure 3.5A, C) or when wild-type neurons were plated on top of $suc$ (xCT-deficient) astrocytes (Figure 3.6). Somewhat to our surprise, we were unable to measure any changes in system $x_c$-mediated cystine uptake in our model system (Figure 3.3). However, this is in accord with findings in mouse 3T3 cells (Sato et al., 2004). Thus, basal activity of system $x_c$- under conditions of energy deprivation appears sufficient to facilitate neuronal injury. This is not unprecedented as the glutamate concentrations needed to kill energy-deprived neurons are far less than those required to kill healthy neurons (Novelli et al., 1988). Given that extracellular levels of glutamate rise, we hypothesize that system $x_c$- activity initiates injury whereas secondary release of intracellular glutamate from dying neurons propagates it (Fogal et al., 2005b). In support, we find that glucose deprivation-induced neuronal injury is positively correlated with neuronal cell density ($r^2=0.85$), with sparse cultures routinely having less neuronal injury than dense cultures (Figure 3.8).
While elimination of astrocytic system $x_c^-$ is sufficient to prevent glucose deprivation-induced neuronal injury, we also found that enhancement of astrocytic system $x_c^-$ could potentiate injury (Figure 3.7B). While IL-1β was utilized as a tool, it is notable that diabetics have increased IL-1β serum levels compared to healthy individuals (Dogan et al., 2006). Given the increasing evidence of crosstalk between the peripheral immune and central nervous systems, it is intriguing to speculate that there may be some physiological relevance.

Overall, our data demonstrate that inhibition of system $x_c^-$ through pharmacological or genetic means is sufficient to dramatically reduce excitotoxic neuronal injury occurring secondary to glucose deprivation. These data highlight the role of astrocytes in non-cell autonomous hypoglycemic neuronal injury and further underscore their potential to serve as therapeutic targets for reducing excitotoxic neuronal injury in vivo.
Figure 3.8. Glucose deprivation-induced injury is dependent on neuronal density. (A) Mixed cortical cultures containing neuronal densities ranging from 1.5-3.5 hemispheres / 10 ml were washed thoroughly into BSS₀. Five and a half (n = 12) or eight hr later (n=6), supernatant was collected for measurement of neuronal cell death. Data are expressed as raw LDH values as a function of neuronal density (A) and percentage of neuronal cell death as a function of raw LDH values (B).
CHAPTER 4

IL-1β-mediated neuroprotection against oxidant stress
4.1 Summary

Astrocytes produce and export the antioxidant glutathione (GSH), which serves as the principal source of substrate for neuronal GSH biosynthesis. Previously, we found that interleukin-1β (IL-1β) enhances the expression of astrocyte system $x_c^{-}$, the transporter delivering the rate-limiting substrate for GSH synthesis — cyst(e)ine. Herein, we demonstrate that IL-1β mediates a system $x_c^{-}$ and time-dependent increase in extracellular GSH levels in cortical astrocyte cultures, suggesting both enhanced synthesis and export. To determine whether this increase could provide protection against oxidative stress in astrocytes and/or neurons, pure astrocyte cultures, as well as, mixed cultures containing both neurons and astrocytes were exposed to tert-butyl hydroperoxide (tBOOH). Astrocytes were incubated with IL-1β (3 ng/ml) or vehicle for 48 hr, after which cells were incubated with tBOOH (0.1-0.7 mM; 2.5 h). tBOOH exposure produced a concentration-dependent injury, which was significantly attenuated by prior IL-1β treatment. Neurons and astrocytes in mixed cortical cultures were also protected from a toxic tBOOH exposure (1.5 mM; 90-120 min) following treatment with IL-1β. Further, astrocyte cultures were exposed to IL-1β (3 ng/ml; 0-24 h) after which mRNA and protein for molecules participating in GSH biosynthesis, export, and metabolism were quantitatively determined. IL-1β enhances mRNA and protein expression of xCT and decreases expression of $\gamma$-glutamyltranspeptidase, the ectopeptidase responsible for extracellular catabolism only. Taken together, these data suggest that IL-1β may increase extracellular astrocytic GSH levels via the enhancement of substrate import.
coupled with the reduction in product catabolism. Hence, while the beneficial
effects of IL-1β are largely underappreciated, we show that IL-1β may be an
important stimulus for increasing astrocyte GSH production, and potentially, total
CNS antioxidant capacity.

4.2 Introduction

Depending on the context, alterations in astrocyte physiology following
neurological injury can promote survival or facilitate death (Liberto et al., 2004;
John et al., 2005). Inflammatory mediators like the cytokine interleukin-1β (IL-1β)
can act on astrocytes to induce changes in gene expression that modify
phenotype and function to produce these dichotomies (Zhao and Schwartz,
1998; Nguyen et al., 2002). For instance, endogenously produced IL-1β appears
necessary for the expression and maintenance of long term potentiation in
hippocampal slices (Schneider et al., 1998; Avital et al., 2003), while higher
concentrations — applied exogenously to mimic pathological expression —
inhibit it (Ross et al. 2003, Avital et al. 2003, Bellinger et al. 1993). Several
studies demonstrate that IL-1β-stimulated astrocytes support neuronal survival
via production of neurotrophic factors (Albrecht et al., 2002; John et al., 2005),
although others demonstrate that IL-1β can interfere with neurotrophin signaling
(Soiampornkul et al., 2008; Tong et al., 2008). Further, IL-1β has been shown to
both exacerbate injury and protect against excitotoxic stimuli [reviewed in
(Pintaux et al., 2009)]. It is hypothesized that these dichotomous effects might
result from cell-specific and/or concentration-dependent actions of IL-1β (Pinteaux et al., 2009).

Additionally, work from our laboratory would suggest the protective versus deleterious effects of IL-1β can be context-dependent. For instance, IL-1β upregulates the activity of the cystine-glutamate amino acid antiporter system $x_c^-$ on astrocytes, which produces excitotoxic neuronal cell death only in the context of energy deprivation (Fogal et al., 2007; Jackman et al., 2010). However, this upregulation is in and of itself not cytotoxic. Interestingly, the same transporter fluxing the glutamate which produces excitotoxicity during periods of energy deprivation has a Janus-face and participates in the uptake of cystine for the synthesis of the neuroprotective antioxidant molecule glutathione (Meister and Anderson, 1983; Bannai and Tateishi, 1986).

Glutathione (GSH) is synthesized from the amino acids glutamate, cysteine, and glycine via two sequential reactions catalyzed by $\gamma$-gammacysteinyl ligase (GCL) followed by glutathione synthetase (GSS)(Meister and Anderson, 1983). Astrocytes release $\approx$10% of GSH/hour into the extracellular compartment (Sagara et al., 1996; Minich et al., 2006), with most GSH being fluxed via multidrug resistance protein 1 (Mrp1)(Hirrlinger et al., 2002). Once in the extracellular space, $\gamma$-glutamyltranspeptidase (GGT1) initiates GSH catabolism, which serves to provide precursors utilized by both neurons and astrocytes for their GSH biosynthetic needs (Dringen et al., 1997; Kranich et al., 1998). In this respect, astrocytes function as indispensible support cells by protecting against oxidative insults (Tanaka et al., 1999; Shih et al., 2003; Jakel et al., 2007) since
both astrocytes and neurons are more susceptible to oxidant-induced injury when astrocytic GSH is deficient (Gegg et al., 2005).

Given the fundamentally protective nature of the inflammatory response, we hypothesized that IL-1β-treated astrocytes could increase GSH. As such, the ability of IL-1β to regulate the metabolic cycle of GSH in astrocytes and the susceptibility of cells to oxidative stress in the presence and absence of IL-1β was evaluated herein.

4.3 Materials and Methods

4.3.1 Cell culture

All media including media stock (MS), glial plating medium, mixed culture plating medium, maintenance medium, and glucose-free balanced salt solution (BSS) have been fully described in section 2.3.1.

Primary astrocyte cultures were derived from cerebral cortices of day 1-3 postnatal CD1 mouse pups (Charles River) as described (Trackey et al., 2001; Jackman et al., 2010).

Mixed cortical cell cultures containing predominantly neurons and astrocytes were prepared by culturing dissociated cells from embryonic day 15 mouse fetuses onto a confluent layer of astrocytes in mixed culture plating media as previously described (Trackey et al., 2001; Jackman et al., 2010). Experiments were performed on mixed cortical cultures after 13-14 days in vitro.
4.3.2 IL-1β Treatment

Astrocytes and mixed cultures were treated with recombinant murine IL-1β (3 or 10 ng/ml respectively; R&D Systems) in an incubation buffer of MS supplemented with 0.1% fatty-acid free BSA (Sigma) for the times indicated in the figure legends. Cells were then returned to a humidified 37°C normoxic (21% O₂) incubator containing 6% CO₂.

4.3.3 Glutathione measurement

Total glutathione [GSx = reduced GSH + glutathione disulfide (GSSG)] concentrations were determined using the GSH-Glo glutathione assay (Promega) per manufacturer’s instruction. Media was collected for analysis of the supernatant fraction and GSH-Glo reaction buffer was added to the cells. Additional lysis buffer was made per manufacturer’s instructions and 200 µL was added per well as a dilution factor to keep relative light units within the dynamic range of the standards. All GSSG within the sample was converted to GSH with the reducing agent TCEP-HCl (final concentration = 1 mM; 10 minutes; room temperature; Thermo Scientific). One hundred microliters of each sample was transferred to a 96-well plate and luciferin buffer was added for 10 minutes with gentle agitation. Luciferase activity was measured in a luminometer (Optocomp II; MGM Instruments). Cellular and tissue culture supernatant levels of GSx were normalized to standards prepared in water and MS, respectively. Standards were linear over the range of 0–5 µM.
4.3.4 Quantitative Real-time PCR (qPCR)

RNA was isolated and first-strand cDNA synthesized as previously described (Jackman et al., 2010). qPCR was performed using mouse-specific primer pairs [Taqman Gene Expression Assays, Applied Biosystems: xCT (Mm00442530_m1), GCLC (Mm00802655_m1), GCLM (Mm00514996_m1), GSS (Mm00515065_m1), Mrp1/Abcc1 (Mm00456156_m1), GGT1 (Mm00492322_m1)] per manufacturer’s instructions. Reactions were run in the Applied Biosystems Fast Real-Time PCR System and relative quantification performed using the comparative cycle threshold method (ΔΔC_T), where C_T values of the transcript of interest were normalized to β-actin C_T values from the same sample, then compared to a calibrator C_T value (untreated cells) to determine the relative fold increase in mRNA. β-actin C_T values were unaffected by IL-1β treatment.

4.3.5 Immunoblotting

Protein expression was determined by Western Blot analysis. Astrocytes in 24-well plates or T25 flasks were washed twice with ice-cold PBS then incubated in lysis buffer [50 mM Tris, pH 8.0, 1.0% Nonidet-P40 (NP40), 150 mM NaCl, and protease inhibitor cocktail (Roche)] for 30 minutes on ice. Cells were harvested by scraping, lysates collected, cellular debris removed by centrifugation (10,000 x g; 15 min; 4°C), and the resulting supernatants stored at -20°C. After thawing, proteins were concentrated via ethanol precipitation. The pelleted protein was resuspended in 1x urea buffer (50 mM Tris, pH 6.8, 2.5%
glycerol, 5% SDS, 4 M Urea, 10 mM DTT, 0.02% bromophenol blue). Thirty µg protein (BCA assay; Pierce) was separated by SDS-PAGE electrophoresis under reducing conditions then electrophoretically transferred to a nitrocellulose membrane (0.2 µm; Bio-Rad). Proteins of interest were detected using species-specific Western Breeze Immunodetection kits (Invitrogen) per manufacturer’s instructions. Primary antibodies were incubated with membranes overnight at 4°C at the following concentrations: α-GGT1 (1 µg/ml; mouse monoclonal; Santa Cruz), α-β-actin (0.3 µg/ml; mouse monoclonal; Sigma) or α-GSS (1 µg/ml; rabbit polyclonal; Abcam). Results were recorded on X-ray film (Fujifilm). Digitized images were analyzed by computer-assisted densitometry (Gel-Pro Analyzer) and GGT1 and GSS protein normalized to their respective β-actin levels.

4.3.6 Tert-butyl hydroperoxide (tBOOH) treatment

Following vehicle or IL-1β exposure, tBOOH (Acros Organics; final concentration: 0.1-1.5 mM) was added to the cultures in an incubation buffer of MS for the times indicated in the figure legends. Experiments were terminated 2.5 - 2.75 hr later by the addition of MTT. Tissue culture supernatant was removed for measurement of lactate dehydrogenase (LDH; see section 4.3.7) immediately before the addition of MTT. In mixed-culture experiments the tBOOH-containing media was replaced with MS at the indicated times (0.5 – 2 hr). Supernatants were collected for measurement of LDH before media replacement and immediately before addition of MTT to assess immediate and
delayed injury. Total neuronal injury was quantified via summation of early and delayed LDH activity measurements.

4.3.7 Measurement of cell death and viability

Cell death was quantitatively determined by spectrophotometric measurement of lactate dehydrogenase (LDH) as described previously (Uliasz and Hewett, 2000). Data are expressed as a percentage of total neuronal or astrocytic LDH activity (defined as 100%) determined by exposing parallel cultures to 250 µM NMDA or 5 µM Calphostin C (Ikemoto et al., 1995), respectively, for 20-24 hr. In mixed culture experiments, cell death values greater than 100% reflect astrocytic LDH release such that all of the neurons and some of the astrocytes are injured. Cell viability was quantified via colorimetric analysis of Thiazolyl blue tetrazolium bromide (MTT; Sigma) reduction as previously described (Lobner, 2000). Briefly, cultures were incubated with 40 µL MTT solution/well (3 mg/ml MTT in PBS; final concentration = 300 µg/ml) for at least 4 h at 37 ºC. The MTT solution was carefully aspirated, crystals were solubilized in acidified isopropanol (90% isopropanol; 10% 1 N HCl; 400 µL/well) and 200 µL was transferred to a 96-well plate. Absorbance at 540 nm was measured with 690 nm background subtraction using a plate reader (SpectraMax M2, Molecular Devices). Viability was expressed as a percentage of maximal MTT reduction (i.e. highest absorbance = 100%) within the plate. Parallel cultures incubated with 5 µM Calphostin C for 24-48 hr defined a complete loss of viability (defined as 0%).
4.3.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (Version 4.03, GraphPad Software, Inc.) as described in each figure legend. Percentage data were first transformed (arcsin square root) before analyses. For qPCR, statistics were performed on the logarithmic retransformation (i.e. geometric means) of \(2^{-\Delta\Delta CT}\) values. In all experiments, data are expressed as the mean ± SEM and significance was assessed at \(p < 0.05\).

4.4 Results

We previously demonstrated in a mixed cortical cell culture system that IL-1β increased system \(x_c^-\) activity, then localized the phenomenon to occur exclusively in astrocytes, not neurons or microglia (Fogal et al., 2007; Jackman et al., 2010). Due to the enhanced delivery of cyst(e)ine via system \(x_c^-\), it was hypothesized that IL-1β would enhance intracellular glutathione levels. Interestingly, following 24 and 48 hr of IL-1β exposure (3 ng/ml), astrocytes exhibit a modest increase in intracellular GSH levels detectable at 24 h (Figure 4.1A). A dramatic increase in extracellular GSH levels is observed at both 24 and 48 h (Figure 4.1B) demonstrating enhanced synthesis and export of GSH. Further, the enhancement in supernatant GSH is dependent on system \(x_c^-\) as this effect is lost when astrocytes are treated concomitantly with IL-1β and 4-CPG (50 µM) but not YM298198 (10 µM) (Figure 4.2B). The former drug inhibits both system \(x_c^-\) and mGluR1α, whereas the latter is a selective mGluR1α antagonist (Fogal et al., 2007), demonstrating that system \(x_c^-\) antagonism alone prevents the
increase in extracellular GSH. Importantly, 4-CPG and YM298198 have no effect on intracellular GSH levels (Figure 4.2A).
Figure 4.1. IL-1β increases supernatant GSH. Pure astrocytes (n = 6 from three experiments) were incubated with IL-1β (3 ng/ml) or vehicle (-IL-1β) for 24-48 hr, after which total intracellular (A) and supernatant (B) glutathione levels were assessed (GSx = GSH + GSSG). Data are expressed as mean ± SEM total GSx (µM) compared to vehicle-treated cells. An asterisk (*) denotes values different from untreated (-IL-1β) astrocytes as assessed by two-way ANOVA followed by Bonferroni’s post hoc test.
Figure 4.2. The IL-1β-mediated increase in supernatant GSH is dependent on system $x_c^-$. Pure astrocytes ($n = 4$ from two experiments) were incubated with IL-1β (3 ng/ml) or vehicle for 24 hr in the presence or absence of 4-CPG (50 µM) or YM298198 (YM; 10 µM), after which intracellular (A) or supernatant (B) GSx levels were assessed. Data are expressed as mean ± SEM total GSx (µM) compared to vehicle-treated (CTL) cells. An asterisk (*) denotes values different from untreated astrocytes, while (#) represents a significant diminution from the IL-1β-mediated enhancement of supernatant GSx levels as assessed by one-way ANOVA followed by Student-Newman-Keul's post hoc test.
Given the interconnectedness between system xcell and cellular GSH levels, the ability of IL-1β to influence other components of the GSH metabolic pathway was assessed. Under basal conditions, the key metabolic transcripts involved in GSH synthesis (GCLC, GCLM, GSS), export (Mrp1), and extracellular catabolism (GGT1) are all expressed in primary cortical mouse astrocytes (Figure 4.3A). Consistent with previous findings, IL-1β (3 ng/ml; 0-24 h) elicits a time-dependent increase in xCT mRNA in astrocytes that peaks at 4 h then slowly declines over time [Figure 4.3B and (Jackman et al., 2010)]. No notable changes in the expression of GCLC, GCLM, or Mrp1 were observed (Figure 4.3C,D,F), and the biological significance of the small (≈1.5-fold), but statistically significant increase in GCLC mRNA remains to be ascertained. The relative expression of GSS mRNA exhibits a time-dependent increase that peaks after 8 hr IL-1β exposure, followed by a decrease and return to baseline levels by 24 hr (Figure 4.3E). Finally, an ≈80% reduction in GGT1 mRNA expression is observed 4 hr after IL-1β treatment which persists until 24 hr, the final time point assessed in these studies (Figure 4.3G). Since we had previously demonstrated that the IL-1β-mediated increase in xCT mRNA was paralleled by an increase in xCT protein expression (Jackman et al., 2010), protein levels of GSS and GGT1 following IL-1β treatment were evaluated. Consistent with the qPCR data, 12-24 hr IL-1β treatment reduces GGT1 protein levels (Figure 4.4), yet this treatment fails to alter GSS protein expression (Figure 4.5).
Figure 4.3. IL-1β regulates xCT, GSS, and GGT1 mRNA expression. (previous page)

(A) Total RNA was isolated from unstimulated astrocytes, reverse transcribed, and PCR performed using specific primers for xCT, GCLC, GCLM, GSS, Mrp1, and GGT1 in separate reactions. (All reactions were for 33 cycles save for GGT1 and β-actin which were for 31 and 23 cycles respectively). (B-G) Pure astrocytes (n=3-6) were treated with IL-1β (3ng/ml) or its vehicle for the indicated durations and relative mRNA expression assessed via qPCR. Data are expressed as mean ± SEM fold change in mRNA compared to untreated cells (0 hr). An asterisk (*) denotes values different from untreated cells as assessed by one-way ANOVA.
Figure 4.4. IL-1β downregulates GGT1 protein expression. Pure astrocyte cultures were incubated with vehicle or 3 ng/ml IL-1β for 12-48 hr. Representative of 5-6 western blots (GGT1). Films were scanned and densitometry performed using Gelpro Analyzer software and GGT1 protein levels were normalized to their corresponding β-actin protein levels and expressed as a fold change (mean ± SEM) relative to control (0 h; set to 1).
Figure 4.5 IL-1β does not alter GSS protein expression. Pure astrocyte cultures were incubated with vehicle or 3 ng/ml IL-1β for 12-48 hr. Representative of 4-6 western blots. Films were scanned and densitometry performed using Gelpro Analyzer software and GSS protein levels were normalized to their corresponding β-actin protein levels and expressed as a fold change (mean ± SEM) relative to control (0 h; set to 1).
It was hypothesized that the IL-1β-mediated changes in GSH metabolism could confer protection against an oxidative stressor. Since catalase cannot neutralize organic hydroperoxides, tert-butyl hydroperoxide (tBOOH) was utilized as its elimination is dependent on GSH and GSH-peroxidase (Dringen et al., 1998; Kussmaul et al., 1999). Depletion of GSH via a 24 hr incubation of astrocytes with buthionine sulfoximine (BSO), a γ-glutamylcysteinylligase inhibitor, can render a sub-lethal concentration of tBOOH (0.3 mM) toxic demonstrating the necessity of GSH for tBOOH elimination in our model system (Figure 4.6). Astrocytes were treated with IL-1β or vehicle for 48 hr then incubated with tBOOH (0-0.7 mM) for 2.5 hr after which cell death and cell viability were evaluated. tBOOH exposure produces a concentration-dependent injury which is attenuated by IL-1β treatment (Figure 4.7), implicating significant glioprotective properties of IL-1β. Finally, mixed cultures were treated with IL-1β (10 ng/ml) or vehicle for 48 hr then incubated with tBOOH (1.5 mM) for 30-120 min, after which a sample of tissue supernatant was collected for LDH analysis, the media was replaced with MS, and injury allowed to progress until 165 min had elapsed at which point MTT was added to the wells to terminate the experiment. Little injury occurs during the acute tBOOH exposure as demonstrated by minor LDH accumulation in the media (gray bars) prior to media replacement. Notably, injury evolves over time. Neuronal injury becomes statistically significant after tBOOH exposure for 60-120 minutes in untreated cultures or after a 120 minute exposure in IL-1β treated cultures (Figure 4.8A). Importantly, neurons are protected against
a 90 and 120 min exposure to tBOOH if they were previously incubated with IL-1β (Figure 4.8A). Further, a 120 min exposure to tBOOH injures both neurons and astrocytes as demonstrated by visual inspection of the astrocyte monolayer and by neuronal cell death percentages greater than 100%. Importantly, pretreatment with IL-1β attenuates the toxicity of tBOOH in mixed cultures at this time point (Figure 4.8B).
Figure 4.6. tBOOH toxicity is GSH-dependent. (A) Pure astrocytes were incubated with BSO (3-300 µM) or vehicle for 24 hr, after which intracellular GSH levels were assessed. Data are expressed as mean ± SEM GSH expressed as a percent of control levels (0 µM BSO). An asterisk (*) denotes values different from untreated astrocytes as assessed by one-way ANOVA followed by Dunnett’s post hoc test. n = 4 from 2 experiments. (B) Pure astrocytes were incubated with BSO (3-100 µM) or vehicle for 24 hr, after which cultures were exposed to a sub-lethal concentration of tBOOH (0.3 mM) for 3.5 hr. Data are expressed as mean ± SEM percent viable astrocytes. n = 6 from three experiments. An asterisk (*) denotes values different from untreated (-tBOOH) astrocytes as assessed by two-way ANOVA followed by Bonferroni’s post hoc test.
Figure 4.7. IL-1β protects astrocytes against tBOOH-mediated oxidative injury. Pure astrocytes were incubated with IL-1β (3 ng/ml) or vehicle (-IL-1β) for 48 hr, after which cells were incubated with tBOOH (0.1-0.9 mM; 2.5 h). (A) Supernatant was collected for LDH analysis of cell death (A) and oxidative injury was terminated with the addition of MTT to assess cell viability. (B) Data are expressed as mean ± SEM percent viable astrocytes. An asterisk (*) denotes values different from untreated astrocytes as assessed by two-way ANOVA followed by Bonferroni’s post hoc test. n = 8-14 from two-three experiments.
Figure 4.7. IL-1β protects against tBOOH-mediated oxidative injury in mixed cultures. Mixed cortical cultures were incubated with IL-1β (10 ng/ml) or vehicle (-IL-1β) for 48 h, after which cells were incubated with tBOOH (1.5 mM) for 90-120 minutes, after which the media was replaced with MS. The experiment was terminated after 165 min when media was collected for assessment of cell death via LDH release (A) and MTT was added to the cultures to assess cell viability (B). Gray bars represent neuronal cell death arising from the acute tBOOH exposure. Data are expressed as mean ± SEM percent neuronal cell death and viability. An asterisk (*) denotes values different from untreated astrocytes as assessed by two-way ANOVA followed by Bonferroni’s post hoc test. n = 4-6 from three experiments.
4.5 Discussion

Maintenance of a reduced intracellular environment is essential to cell survival as altered redox status in astrocytes in favor of pro-oxidant conditions impairs glutamate clearance via GLAST and GLT1 (Trotti et al., 1997), disturbs cell signaling and gene expression, and can damage nucleic acids, proteins, and lipids. Our laboratory has previously published that IL-1β regulates astrocyte system $x_c^-$, a cystine-glutamate antiporter, essential for delivering cyst(e)ine the rate-limiting substrate for the synthesis of the antioxidant molecule GSH (Fogal et al., 2007; Jackman et al., 2010). However, the physiological consequences of the IL-1β-mediated enhancement in system $x_c^-$ were unknown and it remained to be determined whether IL-1β regulated GSH levels in astrocytes as has been shown for other cell types following induction of system $x_c^-$ (Sato et al., 1995a; Sasaki et al., 2002).

Since circulating GSH is unable to penetrate the blood brain barrier (Cornford et al., 1978), GSH within the CNS must be generated in situ. In the CNS, astrocytes function as the predominant provider of GSH and understanding the modulation of GSH levels is of great import. Alterations in GSH content could directly influence astrocytic GSH flux, utilization of GSH precursors by other cell types, and confer resistance against or enhance vulnerability to oxidative insults. In our studies, IL-1β enhanced extracellular levels of GSH and these changes were associated with enhanced expression of xCT and reduced expression of GGT1. Importantly, the IL-1β-mediated changes in astrocytic antioxidant capacity reduces susceptibility to the oxidative stressor tert-butyl hydroperoxide in purified...
astrocyte cultures and in mixed cortical cultures (Figures 4.7, 4.8).

IL-1β and other cytokines have been demonstrated to participate in the regulation of antioxidant levels in non-neural tissue (Kayanoki et al., 1994; Antras-Ferry et al., 1997). In non-neural cells including mouse endothelial cells (Urata et al., 1996) and endometrial stromal cells (Lee et al., 2009), IL-1β increases intracellular GSH levels. Our data are consistent with the previously reported observations that intravenous administration of IL-1β decreases GGT1 activity in the rat hippocampus within 2 h (Kaiser et al., 2006) and in a human astrocytoma cell line following a 24 h exposure to IL-1β (Malaplate-Armand et al., 2000).

The notion that neuroprotection could be mediated by an inflammatory mediator is not unprecedented, as positive effects of IL-1β in the CNS and periphery have already been reported. For example, IL-1β protects mice against lethal challenge via radiation or infection when administered 20-24 hr prior to the insult (Neta et al., 1986; Schwartz et al., 1987; van der Meer et al., 1988). Within the brain, chronic overexpression of IL-1β ameliorates pathology observed in the hippocampus in a mouse model of Alzheimer’s disease (Shaftel et al., 2007). IL-1β enhances neuronal sprouting and/or regeneration in vitro and in the hippocampus and ventral tegmental nucleus in vivo (Fagan and Gage, 1990; Wang et al., 1994; Temporin et al., 2008). IL-1β-deficient mice have impaired blood brain barrier repair compared to their WT counterparts following corticectomy as assessed via extravasation of IgG into brain parenchyma (Herx and Yong, 2001) and remyelination is impaired in these mice secondary to
delayed differentiation/maturation of oligodendrocytes (Mason et al., 2001; Vela et al., 2002). In a similar vein TNF-α, which is induced by IL-1β, promotes oligodendrocyte progenitor maturation and remyelination (Arnett et al., 2001). While the mechanisms are not yet fully elucidated one can speculate that some protective effects of IL-1β can be attributed to transcriptional regulation of genes intimately involved in neuronal and glial proliferation and/or survival including nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) among others (Friedman et al., 1990; Spranger et al., 1990; Albrecht et al., 2002).

It should be noted that a 24 hr exposure of astrocytes to IL-1β alters the expression of approximately 1400 hundred genes (John et al., 2005). In addition to modulating the expression of trophic factors, the beneficial effects of IL-1β may arise via cytokine influence on antioxidant systems. In rat astroglial cultures, LPS-activated microglia induce numerous proteins in astrocytes with antioxidant properties including SOD2, peroxiredoxins, and glutathione S-transferase, and these changes are associated with protection from H₂O₂-induced oxidative stress (Röhl et al., 2008). IL-1β has been demonstrated to alter metallothionein expression in human fetal astrocytes (John et al., 2005). CNTF, a growth factor induced by IL-1β, increases the expression of the astrocyte-specific glutathione-S-transferase (GST-µ) (Levison et al., 1996) important in the conjugation of harmful compounds to GSH and subsequent extrusion from the cell. Additionally, IL-1α regulates MnSOD expression (Mokuno et al., 1994) and IL-1β induces ceruloplasmin (Chang et al., 2001; Kuhlow et al., 2003) -- a copper and iron chelator -- potentially limiting the iron-mediated generation of reactive species via
Given the opposing nature of “typical” pro- and anti-inflammatory mediators, it raises the intriguing possibility that specific cytokines regulate particular components of a system, and that single cytokines or combinations of cytokines may be responsible for the fine-tuning of antioxidant status based on the timing, concentration, and/or kinetics of cytokine expression in specific cell types. In support of this notion, in our studies and those by others IL-1β reduces GGT1 expression/activity, whereas TNF-α can induce GGT1 expression and activity (Reuter et al., 2009). In the liver and liver-derived cell line, IL-1β, and IL-6 can induce Mrp1 mRNA expression (Lee and Piquette-Miller, 2001, 2003; Cherrington et al., 2004), but in primary rat astrocytes TNF-α, but not IL-1β or IL-6, can increase Mrp1 (Ronaldson et al., 2010).

The production of the antioxidant GSH is of obvious importance for the maintenance of cellular health. Although, additional studies are necessary to elucidate the role of IL-1β in post-transcriptional regulation of GSH metabolism, this pleiotropic cytokine unequivocally enhances GSH levels in part via regulation of xCT and GGT1. These findings underscore the importance of understanding the effects of cytokines in various physiological as well as pathophysiological contexts. Ironically, while this pathway allows the CNS to rapidly upregulate GSH in response to an oxidative challenge, it also holds the potential to exacerbate CNS pathology as a consequence of increasing extracellular glutamate levels and the risk of excitotoxicity. The balance between these two possible outcomes must always be kept in mind when considering the physiological consequences
of alterations in system $x_c^-$ expression and function. Indeed, as Shaftel and colleagues articulated so eloquently: “IL-1 can no longer be regarded as simply the villain in the setting of brain injury and disease, but instead might be understood as a factor that can influence the balance between beneficial and detrimental outcomes” (Shaftel et al., 2008). The same is certainly holds true for system $x_c^-$. 
CHAPTER 5

Putative Mechanism of Regulation
5.1 Summary

We recently demonstrated that interleukin-1β (IL-1β) increases system x_c^- activity in mixed cortical cell cultures, resulting in an increase in hypoxic neuronal injury when glutamate clearance is impaired. System x_c^- activity is also deleterious in the context of glucose deprivation in the presence of competent glutamate uptake. Interestingly, when cultures are not under conditions of energy deprivation, the enhancement in system x_c^- by IL-1β produces an accumulation of glutathione in the extracellular space, which can protect both neurons and astrocytes against oxidant-induced injury. Given the context-dependent effects of system x_c^- it is important to understand the mechanisms by which this transporter is regulated. Herein, we demonstrate that NF-κB appears to be a negative regulator with inhibition of NF-κB via BAY-11-7082 enhancing the expression of xCT in a manner that potentiates the effects of IL-1β. The protein kinase A/C/G inhibitor H7 prevents the IL-1β-mediated enhancement in xCT mRNA expression. Interestingly, xCT mRNA expression is enhanced by application of PMA and dBcAMP, activators of PKC and PKA, respectively, but not by 8-Br-cGMP. Finally, the concentrations of H7 were efficacious as it could block the PMA and dBcAMP-mediated enhancement in xCT expression. Overall, our data demonstrate that NF-κB is not involved in system x_c^- regulation by IL-1β and that protein kinase A and/or C likely participates in the regulation of xCT in the presence and absence of IL-1β.
5.2 Introduction

This thesis documents that IL-1β enhances xCT mRNA and protein expression along with the activity of astrocytic system xᵢ⁻ (Chapter 2), yet the mechanism(s) of regulation have not been elucidated. It has been well documented that many agents induce xCT expression and/or system xᵢ⁻ activity including: electrophiles like diethyl maleate (Bannai, 1984a), cystine deprivation and deprivation of other amino acids (Bannai and Kitamura, 1982), oxidative stress via the generation of reactive species (Bridges et al., 2001; Dun et al., 2006), oxygen (Bannai et al., 1989), oxidized low-density lipoprotein (LDL) (Sato et al., 1995b), the HIV tat (transactivation of transcription) protein (Bridges et al., 2004), Aβ (Qin et al., 2006), erythropoietin (Sims et al.), dBcAMP (Gochenauer and Robinson, 2001; Seib et al., in press), and pertinent to this thesis, the inflammatory mediators LPS and TNF-α (Sato et al., 1995a).

Transcriptional regulation of xCT has been demonstrated in non-neural cells (Itoh et al., 1999; Sato et al., 2001; Sasaki et al., 2002; Qiang et al., 2004; Sato et al., 2004) and the xCT 3’ UTR contains numerous AU-rich elements (our unpublished observations), the occupation of which by trans-acting factors is known to influence mRNA stability (Brennan and Steitz, 2001). The xCT promoter contains several cis-acting regulatory elements including four antioxidant response elements (ARE, also referred to as the electrophile response element, EpRE), two amino acid response elements (AARE), and putative AP-1 and NF-κB binding sites (Sato et al., 2000; Sato et al., 2001; Sasaki et al., 2002; Sato et al., 2004). Depletion of GSH facilitates the nuclear
translocation of the transcription factor NF-E2-related factor (Nrf2), which regulates gene expression through its subsequent interaction with AREs (Itoh et al., 1999; Qiang et al., 2004). The transcription factor ATF4 mediates the enhancement of xCT following amino acid deprivation via binding to the AARE (Sato et al., 2004). Other mechanisms by which system $x_c^-$ is regulated remain to be ascertained.

5.3 Materials and Methods

5.3.1 Cell culture

Cell culture media, experimental buffer compositions, and primary astrocyte cultures have been previously described (see section 2.3.1).

2.3.2 Cell Treatment

Cells were treated with recombinant murine IL-1β, dBcAMP, PMA, or 8-Br-cGMP for the times indicated in the figure legends in an incubation buffer of MS supplemented with 0.1% fatty-acid free BSA (Sigma). Cells were then returned to a humidified 37°C normoxic (21% $O_2$) incubator containing 6% $CO_2$.

5.3.3 qPCR

RNA isolation and qPCR have been described in detail previously (see section 2.3.4 and 2.3.5).
5.4 Results

Since signaling in response to IL-1β in astrocytes is preferentially mediated by NF-κB (Srinivasan et al., 2004) and the xCT promoter contains a putative NF-κB consensus sequence (Sato et al., 2001), NF-κB was evaluated as a potential transcriptional regulator of xCT. NF-κB activation was assessed by nuclear translocation of p65 which was rapid and robust, with peak p65 nuclear translocation occurring 15 minutes after IL-1β stimulation then declining over time and returning to baseline levels within 60 minutes (Figure 5.1). Astrocytes were incubated with the NF-κB inhibitor BAY 11-7082, which inhibits phosphorylation of IκB-α (Pierce et al., 1997) for 1 hour after which IL-1β was added to the cultures for 6 h. Importantly, 30 μM BAY 11-7082 is sufficient to block IL-1β-induced translocation of p65 into the nucleus (Figure 5.2A). Inhibition of NF-κB signaling by BAY 11-7082 did not block the IL-1β-mediated enhancement of xCT expression, rather, it potentiated the effects of IL-1β (Figure 5.2B). Similar enhancements in xCT mRNA expression were obtained in experiments utilizing an alternate NF-κB inhibitor PDTC (Appendix Figure A3). These findings are consistent with the observation that low concentrations of LPS induce xCT expression in the absence of NF-κB activation (Sato et al., 2001), suggesting that changes in xCT gene-expression can occur independently of TLR4-mediated NF-κB activation.

Parallel studies were conducted utilizing a variety of inhibitors to identify potential candidates involved in the IL-1β-mediated enhancement of xCT mRNA.
While not an exhaustive screen, the only inhibitor tested that blocked the effect was H7, a pan-protein kinase A/C/G inhibitor (Figure 5.3).

To elucidate which protein kinase has the potential to enhance xCT expression astrocytes were incubated with dibutyryl cAMP (dBcAMP), phorbol 12-myristate 13-acetate (PMA), and 8-Br-cGMP to activate PKA, PKC, and PKG, respectively. dBcAMP and PMA enhance xCT mRNA expression whereas 8-Br-cGMP does not (Figure 5.4). Importantly, the concentration of H7 utilized in the previous experiment is efficacious as it is sufficient to block the IL-1β-, dBcAMP-, and PMA-mediated enhancement in xCT mRNA (Figure 5.5).
Figure 5.1. Time course of NF-κB activation by IL-1β. (A) Pure astrocyte cultures were incubated with vehicle (0 min) or 3 ng/ml IL-1β (15-60 min). Cells were harvested, nuclear and cytoplasmic fractions isolated, and 10 mg protein was separated by SDS-PAGE (12% gel). Western blot analysis was performed using antibodies directed against p65 (NF-κB), β-actin (loading control), and Histone H3 (nuclear marker). Representative of six blots. (B) Films were scanned and densitometry performed using Gelpro Analyzer software. Nuclear p65 protein levels were normalized to their corresponding β-actin protein levels and expressed as a fold increase (mean ± SEM) over control (0 min; set to 1). An asterisk (*) denotes values that are significantly increased over control as assessed by one-way ANOVA followed by Dunnett’s post hoc test of the arc sin square root transformed values (n = 6).
Figure 5.2 NF-κB may negatively regulate xCT expression. (A) Astrocytes were incubated with vehicle or BAY 11-7082 (30 µM) for 30 min after which IL-1β or vehicle was added for 30 min then nuclear accumulation of p65 was assessed via immunocytochemistry. (B) Astrocytes (n=3) were incubated with various concentrations of BAY 11-7082 for 1 hour after which IL-1β or vehicle was added for 6 hr then xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to untreated cells.
Figure 5.3. The pan-PKA/PKC/PKG inhibitor H7 blocks the IL-1β-mediated enhancement in xCT mRNA. Astrocytes (N=2) were incubated with the kinase inhibitors listed for 1 hour after which IL-1β (3 ng/ml) was added for 5 hr and xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to IL-1β-treated cells (IL-1β). Inhibitor concentrations were as follows SB203580, 3µM; SP600125, 30 µM; U0126, 10 µM; PD98059, 50 µM; H89, 1 µM; Calphostin C (CalC), 1µM; KT5823, 1.5 µM; H7, 30 µM; LY294002, 5 µM.
Figure 5.4. PKA and PKC activation induce xCT expression. Pure astrocytes (n=2) were treated with IL-1β (3ng/ml), dBcAMP (500 µM), PMA (100 nM), 8-Br-cGMP (300µM) or its vehicle for 8 hr and xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to untreated cells (CTL).
Figure 5.5. Inhibition of PKA/PKC prevents the IL-1β mediated induction of xCT. Pure astrocytes (n=2) were incubated with H7 (30 µM; 1 hr) after which IL-1β (3 ng/ml), PMA (100 nM) or its vehicle were added for 6 hr or dBcAMP (500 µM) was added for 8 hr and xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to untreated cells (CTL).
5.5 Discussion

This data presented herein suggests that NF-κB is not the transcription factor responsible for the enhancement of xCT mRNA and may actually function as a negative regulator of system \( x_c \). We posit that there may be a constitutively active form of NF-κB in astrocytes, or in a distinct subpopulation of astrocytes, as constitutive NF-κB activation has been reported in mature B cells (Doerre and Corley, 1999), thymocytes (Korner et al., 1991), some thymomas (Hemar et al., 1991) and monocytic cell lines (Griffin et al., 1989). Importantly, constitutive expression of NF-κB has been described in the CNS in some hippocampal and cortical neurons as measured by luciferase reporter assays and immunofluorescence (Kaltschmidt et al., 1994; Bhakar et al., 2002). This notion is plausible as p65 is observed in the nucleus in the absence of stimulation after incubation with the nuclear export inhibitor leptomycin B suggesting that NF-κB shuttles between the nucleus and cytoplasm in astrocytes (Zhai et al., 2004). As such, the role of NF-κB in regulation of xCT warrants further study and astrocytes derived from mutant mice containing a dominant negative form of IκB localized exclusively to astrocytes (GFAP-IκB-DN) or IKK knockout mice might be particularly useful for future inquiries (Li et al., 2000; Herrmann et al., 2005; Zhang et al., 2005).

It should be noted that PMA and PKA can activate NF-κB (Shirakawa et al., 1989; Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990), yet activation
of NF-κB by TNF-α appears to occur in a PKC-independent fashion (Meichle et al., 1990). It has previously been reported that a chronic exposure of astrocytes to dBcAMP for seven to ten days enhances xCT mRNA and protein expression and system $x_c^-$ activity (Gochenauer and Robinson, 2001; Seib et al., in press), yet acute induction of xCT by dBcAMP to the best of our knowledge has not been previously reported. There is also evidence for PKC-mediated regulation of system $x_c^-$ activity levels in neutrophils where xCT mRNA is enhanced after incubation with PMA (Sakakura et al., 2007). However Tang and colleagues demonstrate a PMA-mediated reduction in cystine uptake in rat cortical astrocytes at identical concentrations (100nM) to those utilized in the studies presented herein (Tang and Kalivas, 2003). Further studies are required to understand the discrepancies between Tang’s studies and the data presented in this dissertation.
CHAPTER 6

Conclusion, Discussion, and Future Directions
6.1 Main Findings

The main findings of this dissertation are that: (1) IL-1β enhances system $x_c^-$ activity and xCT mRNA and protein expression in astrocytes, but not neurons or microglia. Further, there is some specificity to this response as IL-1β does not influence expression of the system $x_c^-$ heavy chains (4F2hc or RBAT), LAT2 (the light chain of the system L amino acid transporter), or the predominant glutamate transporters in astrocytes (EAAT1/GLAST and EAAT2/Glt-1). (2) The activity of astrocytic system $x_c^-$ is contributory to neuronal injury following energy deprivation (specifically the IL-1β-mediated enhancement of hypoxic injury and hypoglycemic injury), and (3) astrocytic system $x_c^-$ protects both astrocytes and neurons against cytotoxicity caused by oxidant stress (tert-butyl hydroperoxide).

6.2 Cell specificity: The importance of astrocytes

Astrocytes are the most numerous cell type in the brain and while originally viewed simply as structural support for neurons, we are continuing to learn how astrocyte physiology is critical for the maintenance of homeostasis, in general, and neuronal physiology specifically. In addition to providing structural support, astrocytes provide metabolic support via delivery of energy substrates and buffering of pH and $K^+$, promotion of growth, regulation of synaptogenesis and synaptic maintenance, modulation of neuronal excitability, participation in the formation and maintenance of the blood brain barrier, and regulation of vascular
tone and microcirculation such that functional hyperemia -- the coupling of cerebral blood flow and neuronal activity -- occurs. (Ransom and Sontheimer, 1992; Ullian et al., 2001; Nedergaard et al., 2003; Maragakis and Rothstein, 2006; Markiewicz and Lukomska, 2006; Sidoryk-Wegrzynowicz et al., 2011).

Organizationally, astrocytes only overlap in a narrow interface, such that they are predominantly arranged into non-overlapping domains (Bushong et al., 2002; Halassa et al., 2007b). Despite the non-overlapping organization of astrocytes, there is significant inter-astrocyte communication via a ‘glial syncytium’ which creates an integrated functional unit capable of distributing energy substrates, metabolites, and second messengers widely (Scemes and Spray, 2004). This may be particularly important since astrocytes are a heterogeneous population [for a detailed review see (Hewett, 2009; Zhang and Barres, 2010)] and extensive coupling likely fosters coordinated responses over long distances.

Further, astrocytes participate in gliotransmission via the release of neurotransmitters such as glutamate and other neuromodulators (Nedergaard, 1994; Parpura et al., 1994; Haydon, 2001) enabling bidirectional communication between neurons and astrocytes. This spatial and functional association of the pre- and post-synaptic neurons and the astrocyte processes ensheathing the synapse is referred to as the tripartite synapse (Halassa et al., 2007a). Important in this respect is the finding that a single astrocyte contacts 300-600 neuronal dendrites (Halassa et al., 2007b) and that its foot processes can ensheath up to 100,000 synapses (Bushong et al., 2002; Ogata and Kosaka, 2002). This
structural organization perfectly positions the astrocyte to activate neighboring neurons and influence neurotransmission (Nedergaard, 1994; Parpura et al., 1994).

In addition to releasing glutamate, astrocytes remove glutamate from synaptic and extra-synaptic spaces to reduce the probability of excitotoxic neuronal injury. Glutamate is converted to glutamine, by astrocytic glutamine synthetase (Norenberg and Martinez-Hernandez, 1979) prior to being shuttled to neurons for the re-synthesis of the neurotransmitter glutamate (Danbolt, 2001). In a similar vein, as discussed earlier astrocytes synthesize and export the antioxidant molecule glutathione which is broken down extracellularly and utilized by neurons and glia for cellular antioxidant defense. The protective capacity of astrocytes is remarkable -- with only a small number of astrocytes (<1 % of total cell number) required to protect neurons against an oxidative stress-mediated injury in mixed neuron-glia co-cultures (Shih et al., 2003). Additionally, the ability of astrocytes to support and maintain neuron health may be attributed to the ability of the astrocyte to release numerous growth factors such as NGF, BDNF, PDGF, and more (Raff et al., 1988; Rudge, 1993).

Despite the important homeostatic functions of astrocytes in the developing brain and in the repair of an injured CNS, astrocytic responses can also promote injury. In particular, astrocytes can be activated by glutamate or inflammatory mediators to induce expression and/or release factors that may participate in neuronal injury and/or neurodegeneration. Two mediators include COX2 and NOS2 which have the potential to contribute to neuronal and/or glial
injury (Galea et al., 1992; Simmons and Murphy, 1992; Hewett et al., 1993; Molina-Holgado et al., 2000; Maragakis and Rothstein, 2006).

Results from this dissertation extend the role of astrocytes in both cytotoxicity and neuroprotection. Microarray data shows that a 24 h exposure of human fetal astrocytes to 10 ng/ml IL-1β alters the expression of approximately 1400 genes (John et al., 2005). The alterations in astrocyte gene expression in response to IL-1β may be beneficial or deleterious depending on features such as the concentration, timing, and longevity of release (i.e., acute or chronic enhancement in expression), and the specific cell-type activated.

6.3 Working model for contributions of system $x_{c^{-}}$ to injury and protection (Figure 6.1)

System $x_{c^{-}}$ functions to exchange intracellular glutamate for extracellular cystine. Under physiological conditions, the activity of the transporter does not facilitate the toxic accumulation of glutamate (Baker et al., 2002c; Augustin et al., 2007; Featherstone and Shippy, 2008; Massie et al., 2010b; De Bundel et al., 2011), but rather contributes to basal extracellular glutamate levels and regulates intracellular GSH levels. However, when glutamate uptake is impaired, as it is under hypoxic conditions the increased activity of system $x_{c^{-}}$ via its regulation by the cytokine IL-1β results in the accumulation of extracellular glutamate and subsequent excitotoxic neuronal cell death (Fogal et al., 2007). Our laboratory
postulates that similar alterations in system \( x_c^- \) activity may contribute to neuronal injury \textit{in vivo} following cerebral ischemia in response to the upregulation of IL-1\( \beta \).

To our surprise, system \( x_c^- \) also contributes to excitotoxic neuronal injury under hypoglycemic conditions in the absence of a detectable impairment in glutamate uptake. Further, IL-1\( \beta \) is not required for this toxicity. Thus, it appears that basal levels of glutamate released via system \( x_c^- \) are sufficient to initiate injury when the energy substrate glucose is unavailable. What remains to be elucidated is precisely how system \( x_c^- \) can release sufficient quantities of glutamate that are capable of overwhelming functional glutamate reuptake transporters.

Finally, we have demonstrated that increased transporter activity in mixed cultures under normoxic and normoglycemic conditions does not produce neuronal injury (Fogal et al., 2005a). In fact, in pure astrocytes the enhancement of transporter activity induced by IL-1\( \beta \) actually increases synthesis and export of GSH, producing a net accumulation in extracellular GSH that is associated with neuroprotection.
Figure 6.1 Consequences of system $x_c^{-}$ activity under physiological and pathophysiological conditions. Under physiological conditions (normoxia and normoglycemia; left panel), cystine transported into the cell via system $x_c^{-}$ is rapidly reduced and incorporated into GSH. IL-1β enhances the activity of the transporter, which results in increased delivery of the rate-limiting substrate for GSH synthesis and enhanced export of GSH into the extracellular space which can protect pure astrocytes and mixed cortical cultures against tBOOH-mediated toxicity. Under hypoxic conditions (middle panel), upregulation of system $x_c^{-}$ by IL-1β enhances the delivery of glutamate to the extracellular space. Hypoxia also impairs EAAT/ system $X_{AG}^{-}$ function leading to the net accumulation of glutamate in the extracellular space which results in excitotoxic neuronal cell death. Importantly, system $x_c^{-}$ does not contribute to pure hypoxic neuronal injury (i.e. neuronal cell death occurring under hypoxic conditions in the absence of IL-1β).

In contrast, under hypoglycemic conditions (right panel), the activity of system $x_c^{-}$ expels glutamate into the extracellular space which initiates processes (currently uncharacterized) that lead to the accumulation of extracellular glutamate. The precise mechanism by which hypoglycemic neuronal injury occurs remains to be elucidated, but neuronal cell death occurs independently of alterations in EAAT/ system $X_{AG}^{-}$ activity and synaptically-derived neuronal glutamate. [Based on results described herein as well as those found in (Fogal et al., 2005a; Fogal et al., 2007).]
6.4 Hypoglycemic neuronal injury in the presence of competent glutamate reuptake

What remains to be determined is precisely how system $x_c^{-}$ induces neuronal injury in the absence of impaired glutamate uptake as assessed via $^3$H-D-aspartate uptake. This finding is consistent with the literature (Swanson and Benington, 1996; Bakken et al., 1998), and estimates of glutamate reuptake transporter expression suggest that 2,500 – 10,000 transporters per $\mu m^3$ are expressed in the brain (Bergles and Jahr, 1997; Lehre and Danbolt, 1998). These high-affinity, highly expressed glutamate transporters should be able to efficiently remove the glutamate that is fluxed through system $x_c^{-}$, especially given the fact that glutamate transporters could theoretically reduce extracellular glutamate to a concentration of $\approx 2$ nM (Zerangue and Kavanaugh, 1996; Cavelier et al., 2005). Based on studies by Wyatt and colleagues, heteroexchange via system $x_c^{-}$ is predicted to increase extracellular glutamate at a maximal rate of 0.6 $\mu$M/s (Cavelier et al., 2005), which is critically dependent on extracellular cystine concentrations, the levels of which are disputed [estimates range from 0.13 $\mu$M reported in the nucleus accumbens (Baker et al., 2003), to 100-200 $\mu$M which is routinely used in tissue culture studies (Murphy et al., 1989; Fogal et al., 2005a; Fogal et al., 2007; Jackman et al., 2010)]. This begs the question are there additional cellular sources of glutamate that contribute to injury or are the techniques employed in our study not sensitive enough to detect changes in glutamate uptake?
As mentioned previously, *in vivo* deafferentation experiments and *in vitro* studies utilizing tetanus toxin implicate neuronal release of glutamate as contributory to hypoglycemic neuronal injury (Wieloch et al., 1985; Monyer et al., 1992). Yet in our hands, a concentration of tetanus toxin (300 ng/ml) that cleaves neuronal synaptobrevin-2 and blocks depolarization-induced glutamate release (Taylor and Hewett, 2002; Fogal et al., 2005b) — fails to attenuate neuronal injury in this paradigm (Figure 3.1D). Further experimentation revealed that when 3 µg/ml tetanus toxin (the highest concentration utilized by Monyer and colleagues) was applied to our mixed cultures one day prior to glucose deprivation there was some protection (15% neuronal protection), but it does not recapitulate the robust finding of 40% neuronal protection reported in their studies (Monyer et al., 1992). It suggests that there is a small contribution of neuronally-derived glutamate to injury, yet still leaves room to consider additional mechanisms of astrocytic glutamate release as participatory in hypoglycemic neuronal cell death.

6.5 Future Directions

Logical future directions include elucidation of the molecular mechanisms by which IL-1β regulates system x_c^- in astrocytes and to determine whether PKA and PKC are involved in this process. Clearly, the finding that NF-κB inhibitors enhance the expression of xCT mRNA is intriguing and deserves further attention. Similarly, Nrf2, a known regulator of astrocytic xCT and other antioxidant molecules (Sasaki et al., 2002; Lewerenz et al., 2009b), that requires
PKC-mediated phosphorylation for activation (Huang et al., 2002; Numazawa et al., 2003) should be examined closely as a candidate transcription factor regulating the processes described herein.

Macroscopically, the biological relevance of this transporter to pathology in the entire organism needs to be evaluated. As such, in vivo studies should be carried out to characterize the role of energy deprivation-induced injury in sut/sut mice following middle cerebral artery occlusion (MCAO) as a model of cerebral ischemia and determine whether insulin-induced hypoglycemia in sut/sut mice recapitulates the in vitro hypoglycemia findings. In vivo oxidative stress models should be utilized to determine whether IL-1β can enhance GSH and/or confer protection in vivo. However, discrepancies between in vivo and in vitro findings may arise because animals have compensatory mechanisms which allow them to thrive, whereas in vitro preparations require functional system x_c^- activity or a reducing agent such as β-ME for survival. For example, one might hypothesize that the loss of system x_c^- function would render animals more sensitive to neuronal injury following MCAO due to reduced GSH levels. In fact, preliminary data suggests the opposite and sut/sut mice appear to be protected against ischemic injury (Figure 6.2). Interestingly, xCT-null mice do not have reduced tissue GSH levels (Sato et al., 2005; Massie et al., 2010b; De Bundel et al., 2011), although plasma GSH levels are reduced (Sato et al., 2005). Moreover, recent work by Massie and colleagues demonstrates that the loss of system x_c^- does not decrease striatal or hippocampal GSH levels and actually protects neurons against 6-hydroxydopamine-induced injury and elevates the threshold
for limbic seizures induced by intravenous infusion of kainate (an ionotropic kainate glutamate receptor agonist) and pilocarpine (a muscarinic cholinergic receptor agonist) or intraperitoneal administration of NMDA (an ionotropic glutamate receptor agonist) in mutant mice (Massie et al., 2010b; De Bundel et al., 2011). This paradoxical finding is attributed to the decrease in glutamate release via system $x_c^-$, a mechanism that would also produce protection in the setting of ischemia.
Figure 6.2. Comparison of ischemic brain damage between wild-type and sut/sut mice. (A) Wild-type (WT; left) and sut/sut mice (right) were subjected to 45 minute reversible MCAO and sacrificed three days later. Brains were processed using TTC staining as previously described (Fogal et al., 2007). The lack of TTC staining (white areas, save for the corpus callosum) represents infarcted tissue. Representative images of TTC-stained coronal brain sections (2mm) from 2 animals of each genotype are depicted. (B) Quantification of infarct size in the affected hemisphere (HEMI), cortex, and caudate-putamen (CP) is shown (n = 4 per genotype).
6.6 System $x_c^-$ as a therapeutic target

System $x_c^-$ is an important biological transporter that has already been utilized as a therapeutic target in the clinics. Sulfasalazine is a system $x_c^-$ inhibitor utilized for the treatment of inflammatory bowel diseases including Crohn’s disease and ulcerative colitis, and rheumatoid arthritis (Linares et al., 2011). In the immune system, the proliferation of T cells is regulated by antigen-presenting cells such as dendritic cells and macrophages via the availability of the amino acid cysteine (Gmunder et al., 1990; Angelini et al., 2002) because T cells lack system $x_c^-$ (Bannai, 1984b; Gmunder et al., 1991). Hence, reducing cysteine delivery to T cells by inhibiting system $x_c^-$ might be a useful strategy to limit lymphocyte activation and related pathology in T-cell mediated autoimmune diseases. Further, sulfasalazine and other system $x_c^-$ inhibitors show promise as adjuvant chemotherapeutics in certain cancers, including gliomas (Chung et al., 2005; Lo et al., 2008; Savaskan et al., 2008; Chung and Sontheimer, 2009; Pham et al., 2010). Whether inhibition of system $x_c^-$ can limit excitotoxic neuronal degeneration in humans remains to be ascertained, but preliminary experiments in a model of experimental ischemia seem promising (see section 5.5) especially in light of the findings that brain GSH levels are not compromised by loss of system $x_c^-$ function in vivo (Massie et al., 2010b; De Bundel et al., 2011).
6.7 Overall significance

Neuroinflammation is a feature of a variety of neurological diseases and disorders. While traditional views asserting that inflammation and the inflammatory mediator IL-1β contribute to disease pathology in the CNS are recapitulated in our model of inflammatory hypoxic neuronal injury, the data contained in this dissertation demonstrate that IL-1β promotes GSH synthesis and export which serves to protect both neurons and astrocytes against oxidative injury. Understanding the regulation of system $x_c^-$ by IL-1β could help to devise strategies to harness the beneficial effects of system $x_c^-$ to increase intracellular GSH, and when appropriate, to reduce its activity to decrease the probability of excitotoxic neuronal injury. Knowledge of the mechanisms by which one can modulate system $x_c^-$ activity and the resultant effects on neural physiology and pathophysiology will continue to inform researchers and clinicians of the diverse contributions of astrocytes and inflammation to health and disease states and may guide the design of novel therapies that target astrocytes rather than neurons.
Appendix
Supplemental Figure A1. *Sut/sut* astrocytes require β-mercaptoethanol (β-ME) to support growth. Primary astrocyte cultures were prepared from *sut/sut* mice as described in the methods. One half of the cells were cultured in astrocyte plating media that was supplemented with β-ME (55 µM), whereas the other half received standard astrocyte plating media. Cells were incubated for eight (A) or fifteen (B) days, after which MTT (3 ng/ml in PBS) was added to the wells to assess cell viability (as described in section 4.3.7). Cell viability was normalized to acidified isopropanol alone (defined as 0 % cell viability). n = 12.
Supplemental Figure A2. Hypoxic neuronal cell death is reduced in cultures containing *sut/sut* astrocytes derived from single pup dissections. Chimeric mixed cortical cell cultures were obtained by plating WT neurons on astrocytes derived from *sut/sut* mice (black bars). These and control cultures (WT neurons on WT astrocytes; white bars) were treated with 3 ng/ml IL-1β or vehicle for 20-24 hr, washed, and then deprived of oxygen for 4-5 hr. The percentage of total neuronal cell death was determined 20-24 hr later (n = 6-9). An asterisk (*) indicates a significant within-group difference, while a pound (#) sign indicates a significant between-group difference as determined by a two-way ANOVA followed by Bonferroni’s post hoc test. Significance was set at p < 0.05.
Supplemental Figure A3. Additional evidence that NF-κB may negatively regulate xCT expression. Astrocytes (n=2) were incubated with various concentrations of the NF-κB inhibitor, PDTC for 1 hour after which IL-1β or vehicle was added for 6 hr then xCT mRNA assessed via qPCR. Data are expressed as mean ± SEM fold change in xCT mRNA compared to IL-1β-treated cells (set at 1).
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