The sensitivity of glutamate transport to the depletion of glutathione

Todd M. Seib

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THE SENSITIVITY OF GLUTAMATE TRANSPORT TO THE DEPLETION OF GLUTATHIONE

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Submitted in partial fulfillment of the requirements for the

Degree of Master of Science

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Abstract

The amino acid glutamate is known to mediate both excitatory synaptic signaling and receptor-mediated excitotoxicity in the mammalian CNS. Excitotoxic insults produced by excessive levels of glutamate are thought to trigger a number of deleterious pathways, including ion-induced osmotic swelling, Ca$$^{2+}$$-mediated pathology, and the production of reactive oxygen species (ROS). Therefore, the efficient regulation of glutamate in the extracellular environment by neuronal and glial glutamate transporters is fundamental to the prevention of neuronal injury and neurodegeneration. Previous studies have shown that excitatory amino acid transporters (EAATs) are sensitive to oxidative inactivation by exogenous ROS. The vulnerability of these transporters may represent an important link between the pathological processes of excitotoxicity and oxidative stress. In the present study, the sensitivity of glutamate transport to mild oxidative stress induced by chronic glutathione depletion was examined in long-term cultures of primary astrocytes. Pharmacology and immunoblotting experiments indicated that the EAAT expressed in the astrocyte cultures was the GLAST subtype. To assess the sensitivity of the EAATs to endogenous levels of ROS produced under normal conditions, intracellular GSH was depleted with buthionine sulfoximine (BSO) and the rate of D-aspartate transport measured. GLAST activity did not change in the astrocytes chronically depleted of GSH, suggesting that GLAST was insensitive to oxidative inactivation under these conditions. The sensitivity of transport activity was then examined by exposing cells to a non-lethal level of exogenous oxidants. While transport activity decreased following the mild oxidant challenge, there was not a significant potentiation of this effect in cells treated with BSO. Tryptophan fluorescence was also measured as a marker of oxidative damage to protein, although, neither chronic GSH-depletion nor the acute mild ROS treatment produced a significant change. This suggested that under the conditions used, loss of transport activity may be a more sensitive indicator of oxidative stress than more general markers of protein oxidation. We conclude that while glutamate transport is sensitive to oxidative stress, GSH may not be important for protection from ROS. The relationship between other antioxidant systems and glutamate transport activity requires further investigation to more fully understand the pathological mechanisms involved in EAAT dysfunction.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AB</td>
<td>Assay buffer</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSO</td>
<td>L-Buthionine sulfoximine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DHK</td>
<td>Dihydrokainate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>EAAC1</td>
<td>Excitatory Amino Acid Carrier 1</td>
</tr>
<tr>
<td>EAATs</td>
<td>Excitatory Amino Acid Transporters</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate/Aspartate Transporter</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glial L-glutamate transporter 1</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative Fluorescence Units</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>X/XO</td>
<td>Xanthine/Xanthine Oxidase</td>
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The research presented in this thesis investigates the vulnerability of the glutamate transporters in the central nervous system (CNS) to oxidative damage and the role that glutathione may play as an oxidative protectant. As will be discussed, the glutamate transporters play a critical role in regulating extracellular glutamate levels in the brain and an attenuation of their activity by oxidative stress could increase the likelihood of glutamate-mediated neuropathology. Therefore, the sensitivity of the glutamate transporters may represent a significant link between two major pathological pathways known to contribute to brain damage: glutamate-mediated neurodegeneration (excitotoxicity) and free-radical-mediated oxidative damage. For this reason, the introduction to this thesis will review:  

i) glutamate as an excitatory neurotransmitter, its role in excitotoxicity, and its regulation by the glutamate transporters
ii) reactive oxygen species formation and oxidative stress, the susceptibility of the CNS, and protective mechanisms, and
iii) the potential link glutamate transport plays between excitotoxicity and oxidative stress.

**Glutamate as an Excitatory Neurotransmitter**

Glutamate is now widely accepted as the major excitatory neurotransmitter in the mammalian CNS and is thought to be responsible for most fast synaptic transmission, as well as contribute to developmental plasticity, and higher cognitive function (memory, learning) (Cotman et al., 1995). The nature of glutamate’s excitatory action on brain tissue was first confirmed in 1954 by Hayashi, and since then, interest in glutamate
neurotransmission has continued to grow and intensify as accumulating evidence suggests its role not only in normal synaptic communication, but also in the development of neurodegenerative disease (e.g. ALS, Parkinson’s, Alzheimer’s), traumatic neuronal injury damage, tumor development, and infectious disease (e.g. HIV) (Lipton et al., 1994; for review see Danbolt, 2001). However, glutamate is not a newly discovered molecule, even though its role as a neurotransmitter may be. Glutamate is an amino acid, acting as a metabolite in various cellular reactions (e.g. protein synthesis, glutamine synthesis, glutathione synthesis, and energy metabolism). Glutamate is also highly distributed throughout the CNS. These features are not common to other well-characterized neurotransmitters (GABA, dopamine, seratonin, norepinephrine, etc.) and therefore glutamate’s role as a neurotransmitter was initially questioned. Over a period of about thirty years studies have established that glutamate exhibits all the necessary hallmarks of a classical neurotransmitter: (1) glutamate exists in the tissues of the CNS at a concentration of about 5-15 mmol per kg wet weight depending on the region (Schousboe, 1981), (2) exogenously applied glutamate has the ability to activate post-synaptic receptors in a manner similar to that of endogenous transmitter, (3) it is sequestered into synaptic vesicles via an energy dependent mechanism driven by an electrochemical proton gradient and (4) released in a Ca^{2+} dependent manner upon depolarization of pre-synaptic membrane (for review see Ozkan et al., 1998), and finally (5) a mechanism to terminate signal transmission exists (i.e. transport) (for review see Danbolt, 2001).
**Excitatory Amino Acid Receptors**

When an action potential propagates down the axon of a glutamatergic neuron, Ca\(^{2+}\) entry through voltage-gated calcium channels stimulates the fusion of glutamate-containing synaptic vesicles, and glutamate is released into the synaptic cleft. Upon release, glutamate can potentially bind to a number of receptors, including post-synaptic ion-coupled receptors, the activation of which allows the excitatory signal to continue.

Glutamate receptors can be divided into two main subtypes, ionotropic and metabotropic.

The ionotropic receptors, which are located pre and post-synaptically, bind glutamate and induce post-synaptic depolarization by gating voltage-dependent and voltage-independent currents carried by Na\(^+\), K\(^+\), and Ca\(^{2+}\) (for review see Monaghan et al., 1989). The three subtypes of ionotropic receptors are pharmacologically distinct and were initially characterized by and named after specific agonists (N-methyl-D-aspartate, NMDA; kainic acid, KA; and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, AMPA). The NMDA receptor is both ligand and voltage gated, allowing Ca\(^{2+}\) and Na\(^+\) influx through its channel. Aside from the binding site for L-glutamate, there are at least four other pharmacologically distinct binding domains on the receptor. Among them are: 1) a glycine binding site, 2) a site within the channel that binds phenylcyclididine and related compounds, 3) a voltage-dependent Mg\(^{2+}\) binding site, and 4) an inhibitory site that binds Zn\(^{2+}\) and other divalent cations. At the resting potential, the binding of Mg\(^{2+}\) within the NMDA receptor channel effectively blocks the receptor. The influx of Ca\(^{2+}\) through the NMDA receptor can occur only after membrane depolarization (often by Na\(^+\) influx through the non-NMDA ionotropic receptors) relieves the Mg\(^{2+}\) blockade.
Ca²⁺-influx through NMDA is linked to a number of downstream signaling pathways including PLA₂ activation, the production of reactive oxygen species, cGMP activation, and phosphorylation of transcription factors (Farooqui et al., 1994). Through these and other yet undefined post-synaptic mechanisms, NMDA receptors are thought to contribute significantly to plasticity in the developing and mature brain. NMDA receptor activation is the pivotal event in the recruitment of additional receptors and the amplification of post-synaptic signal in the process of long-term potentiation (LTP) (Monaghan et al., 1989). LTP is characterized by increases in post-synaptic potentials following high-frequency afferent stimulation of a discrete circuit (Collingridge et al., 1987). The post-synaptic events in LTP are particularly noteworthy, however, in that synaptic transmission within the stimulated circuit is enhanced over the long term (minutes to days), representing the first potential molecular mechanism underlying learning and memory.

The second receptor subtype, the metabotropic receptors (mGluRs), are located both pre- and post-synaptically and act as G-protein coupled receptors linked to phospholipase C activation and both activation and inhibition of the adenylate cyclase. At least six different subtypes of mGluRs have been identified and are classified into three subgroups based on sequence and pharmacology (Cotman et al., 1995). The continued development of conformationally constrained glutamate analogs (1-aminocyclopentane-trans-1, 3-dicarboxylate, trans-ACPD; and 2-amino-4-phosphonobutyrate, AP4) that are inactive at the ionotropic receptors and exhibit pharmacologically distinct activity between the subgroups, can serve as tools to further differentiate mGLuR subtypes.
EXCITOTOXICITY

A shared mechanism exists through which normal function and toxicity can occur in glutamate neurotransmission. This mechanism is primarily mediated by the ionotropic receptors. As previously mentioned, glutamate stimulates ionotropic receptors as a necessary part of normal signal transmission. Paradoxically, the ionotropic receptor subtype also mediates the process through which endogenous glutamate can be toxic. Excitotoxicity is a process whereby excessive levels of glutamate can over-stimulate the ionotropic receptors leading to osmotic swelling, Ca^{2+} influx, and production of reactive oxygen intermediates, which can eventually result in neuronal cell death (Choi, 1988; Dugan et al., 1994; Farooqui et al., 1994; Lipton et al., 1994; Zeevalk et al., 1998).

Currently, it is accepted that there are two main mechanisms of neuronal damage associated with excessive depolarization from high glutamate concentrations; osmotic damage from ion-gradients, and Ca^{2+} related damage (Choi, 1985; Choi, 1987).

The first mechanism, and the most acute, is caused by the influx of Na^{+} ions through ionotropic receptors following depolarization. Consequentially, Cl^- ions enter the cell to restore charge equilibrium and water influx occurs as the cell becomes hypertonic. Additionally, increased ATP consumption from activated Na^{+}/K^{+} ATPases may occur in response to increased Na^{+} permeability. Eventually, neuronal swelling results, leading to cell death. This mechanism is dependent upon extracellular Cl^- and Na^{+} ion availability, can be mimicked by high amounts of K^{+}, and may be less significant in vivo than in in vitro preparations (Choi, 1987). A second, more chronic component of glutamate neurotoxicity is observed and shown to be Ca^{2+}-dependent (Choi, 1987).
Figure 1. **The glutamatergic synapse.** Intracellular glutamate in the pre-synaptic terminal is sequestered into vesicles through the vesicular glutamate transporter (GVT). Following membrane depolarization voltage-dependent calcium channels open and Ca\(^{2+}\) influx stimulates the fusion of glutamatergic vesicles with the membrane. The release of glutamate into the extracellular space results and through receptor-mediated depolarization, the excitatory signal continues. Both ionotropic and metabotropic excitatory amino acid receptors are identified pharmacologically with selective agonists (i.e. NMDA, N-methyl-D-aspartate; KA, kainic acid; and AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ACPD 1-aminocyclopentane-trans-1, 3-dicarboxylate). The EAATs (acronym for excitatory amino acid transporter cloned from human) are Na\(^+\)-dependent high-affinity transport proteins that remove glutamate from the extracellular space and serve to terminate the excitatory signal. System X\(_{\text{C}}\) (xCT/4F2hc) exchanges extracellular cystine for intracellular glutamate.
Not surprisingly, the NMDA receptor became an attractive candidate as the primary mediator of the Ca\(^{2+}\) dependent component of glutamate neurotoxicity (for review see Choi, 1988). The initial observation that Ca\(^{3+}\)-free conditions were neuroprotective for neurons exposed to toxic amounts of glutamate (0.5mM) tied Ca\(^{2+}\) to glutamate, a requisite beginning for NMDA involvement (Choi, 1985; Choi, 1987). More convincing evidence came, however, when investigators demonstrated that Mg\(^{2+}\) (known to serve as a voltage block to NMDA receptors) was also capable of blocking the late Ca\(^{2+}\) effect (Choi, 1987). To support these findings, Murphy and colleagues demonstrated that NMDA induced increases in intracellular Ca\(^{2+}\) levels, a process that was both sensitive to Mg\(^{2+}\) and insensitive to La\(^{3+}\) and Li\(^{+}\) (Murphy et al., 1987). This finding suggests that increases in intracellular Ca\(^{2+}\) are mediated primarily by influx through the NMDA receptor and less significantly through voltage-gated channels activated by membrane depolarization. Pharmacological studies then provided strong evidence for NMDA involvement in excitotoxicity when investigators found that selective NMDA antagonists (2-amino-5-phosphonovalerate, ketamine, APH) effectively attenuated glutamate neurotoxicity, while non-NMDA antagonists had less effect (Choi et al., 1988).

It is now accepted that increased calcium influx through the NMDA receptor is the second (and longer-term) consequence of glutamate-induced excitotoxicity. The implications of this statement can be profound. Under normal circumstances, intracellular Ca\(^{2+}\) acts beneficially as a second messenger. However, sustained increases in intracellular Ca\(^{2+}\) can over-activate enzyme systems (e.g. phospholipase A\(_2\), neuronal NOS, proteases, and calcium/calmodulin complexes) which can lead to impairment of mitochondrial oxidative phosphorylation, depletion of energy reserves, reactive oxygen
species formation, and proteolysis (Choi, 1988; Dugan et al., 1994; Ciani et al., 1996; Leist et al., 1997; Halliwell, 2001). The conclusion is that excitotoxicity arises from endogenous stores of glutamate and is mediated through the identical mechanism as normal neurotransmission, namely, EAA receptor activation. Clearly, one of the most significant threats to the CNS is that which lies within, as neurodegeneration is the endpoint for glutamate-induced excitotoxicity (Choi, 1988).

The excitotoxic potential of glutamate, accompanied with it’s high levels in the CNS, has made it a prime candidate in a number of pathologies, including acute damage in stroke, anoxia, and trauma as well as chronic neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer’s, and Parkinson’s disease. It follows then that the efficient regulation and removal of glutamate from the synaptic cleft is required to insure the delicate balance between normal neurotransmission and neuropathology. While more than one protein family has the ability to transport glutamate, it is generally accepted that the majority of glutamate regulation in the CNS occurs through the action of high affinity, sodium-dependant, excitatory amino acid transporters (EAATs) (Robinson et al., 1997).

**L-GLUTAMATE TRANSPORT**

Within milliseconds following release from the presynaptic nerve terminal into the synaptic cleft, glutamate is moved intracellularly through the glutamate transporters (Clements et al., 1992). The efficient removal of L-glutamate occurs continuously and establishes an intracellular store of glutamate several thousand-fold higher than that in the extracellular space (depending on region the brain contains 5 -15 mM per kg wet weight
Figure 2. Na⁺-dependent excitatory amino acid transport.
but only 3-10 μM is found extracellularly) (for review see Danbolt, 2001). The transport of glutamate is thermodynamically unfavorable and occurs via secondary active transport. The energy barrier of glutamate transport is overcome by coupling it with the exergonic flow of sodium into and potassium out of the cell down their chemical and electrical gradients originally established by a Na⁺/K⁺ ATPase. Inhibition of Na⁺/K⁺ ATPase with ouabain uncouples ATP driven flow of sodium and potassium against their gradients and as a result their exergonic flow back down their gradients can no longer be harnessed for secondary active transport and glutamate uptake decreases (and may possibly reverse) (Volterra et al., 1994). The stoichiometry of glutamate transport (Figure 2) is believed to involve the co-transport of three sodium ions and a proton in exchange for one potassium ion for each molecule of glutamate transported (at physiological pH L-glutamate has a net negative charge) resulting in an electrogenic process (net positive charge transported) (Zerangue et al., 1996). A non-thermodynamic chloride conductance is thought to be associated with transport as well (Fairman et al., 1995).

Following transport into neurons and glia, glutamate is subject to metabolic reactions, the most important of which are catalyzed by L-glutamate decarboxylase (GAD), glutamic-oxaloacetic transaminase (GOT), glutamate dehydrogenase (GLDH), glutamine synthetase, and glutaminase. The two most common fates of glutamate are (1) conversion to glutamine by glutamine synthetase (following transport into glial cells) and (2) conversion to α-ketoglutarate through the actions of GLDH or GOT. Glutamine can be transported from astrocytes into neurons and then converted back to glutamate for synaptic release. This process, known at the glutamine-glutamate cycle, allows
glutamine to contribute to glutamate recycling by serving as a non-excitotoxic precursor (does not bind to glutamate ionotropic receptors) and acts as a secondary intracellular glutamate pool (Schousboe, 1981; Danbolt, 2001). α-Ketoglutarate can enter oxidative metabolism through the TCA cycle or be converted back to glutamate through aminotransferases or by GLDH catalyzed reactions running in reverse.

Just as the glutamate-initiated excitatory signal is mediated through a number of receptors, the process of signal termination is dependent upon multiple proteins. In 1992 the existence of multiple glutamate transporters was confirmed with the isolation of cDNA clones from rat brain (GLT-1 and GLAST) and rabbit intestine (EAAC1) (for review see Robinson et al., 1997). Antibodies raised against the C-terminal domains of each transporter detect 65-73 kDa proteins in immunoblots and have been used to identify the cellular distribution of the transporters in rat CNS (Rothstein et al., 1994). GLT-1 and GLAST are responsible for the majority of activity attributed to the glutamate transporters in the mammalian CNS (Robinson et al., 1997; Danbolt, 2001). Of the known subtypes, GLT-1 is the most predominant transporter throughout the brain and is primarily expressed on astrocytes in the normal adult rat CNS. Neuronal expression of GLT-1 is thought to be restricted to neurons of the retina even though GLT-1 mRNA has been found in certain populations of neurons. The regional distribution of GLAST is less widespread than GLT-1, however in the cerebellum it is the major glutamate transporter. Unlike GLT-1, GLAST protein and mRNA appear to be exclusively localized to glia. The concentration of EAAC1 in the brain is lower than GLAST and GLT-1 and thus information on EAAC1 localization is less well known. It is generally agreed that EAAC1 has a wide neuronal distribution in the mammalian brain in both glutamatergic
and GABAergic neurons with the highest levels of EAAC1 protein expressed in the hippocampus, cerebellum, and basal ganglia (for review see Danbolt, 2001).

Five sodium-dependent glutamate transporters have been identified and cloned in the human CNS and have been named excitatory amino acid transporters 1-5 (EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5) (for reviews see Rothstein et al., 1994; Danbolt, 2001). Based on sequence homology, EAAT1 is the human homolog of GLAST, EAAT2 is the homolog of GLT-1, and EAAT3 is homologous to EAAC1. Among the subtypes, it is accepted that L-glutamate, L-aspartate, and D-aspartate serve as substrates (Arriza et al., 1994; Robinson et al., 1997). Transport-mediated current measurements, radiolabeled uptake, and inhibition studies now indicate that cysteine can also serve as a substrate for the glutamate transporters (predominantly EAAT3) (Zerangue et al., 1996; Chen et al., 2003).

The exact topology of the glutamate transporters is not currently agreed upon and further delineation is necessary. A consensus exists, however, that common to the subtypes are at least six α-helical transmembrane domains with a long extracellular stretch between TM3 and TM4 and that both the N- and C-terminals are intracellular (Danbolt, 2001). The glutamate transporters, apart from regional, cellular, and subtype distinctions, exhibit yet another level of complexity. Freeze-fracture electron microscopy and electrophysiological studies in oocytes now indicate that the glutamate transporters may also function as oligomers, consisting of as many as five symmetrical domains (Eskandari et al., 2000).

The EAATs and their homologues are not the only proteins capable of translocating glutamate across the plasma membrane in the mammalian CNS. Bannai first
described a transporter that mediated a one-to-one exchange mechanism for glutamate and cystine in fibroblasts, now referred to as system $X_c^-$ (Bannai, 1986). Unlike the EAATs, this exchange process is Na$^+$-independent and under normal conditions transports glutamate out of the cell, rather than sequestering it intracellularly. Bannai demonstrated that glutamate and cystine can serve as substrates for exchange of themselves or the other on both sides of the membrane (Bannai, 1986). Despite the competitive nature of exchange, the intracellular glutamate levels are much higher than cystine and dictate that the system exchanges an intracellular glutamate for an extracellular cystine under normal physiological conditions (Bannai, 1986; Cho et al., 1990).

**The Presence of Reactive Oxygen Species**

The fidelity of glutamate transport can be compromised through a number of pathological mechanisms, potentially resulting in excessive glutamate levels and excitotoxicity. The production of ROS is one mechanism that may adversely affect glutamate transport. Reactive oxygen species include free radicals and reactive non-radicals. Free radicals are species containing one or more unpaired electrons. A broad range of compounds fall under this definition, including superoxide radical ($O_2^*$), hydroxyl radical ($\cdot$OH), and nitric oxide (NO$^*$). Technically, molecular oxygen ($O_2$) could be included as well due to the two unpaired electrons in the 2p orbital. Reactive non-radicals include hydrogen peroxide ($H_2O_2$), peroxynitrite (ONOO$^-$), and singlet oxygen ($^1O_2$). ROS are generally very reactive and can easily abstract hydrogen atoms and electrons from important macromolecules (proteins, lipids, carbohydrates).
A major source of ROS in respiring cells can be attributed to the incomplete reduction of molecular oxygen by mitochondrial cytochrome oxidase enzymes (Figure 3). As a consequence, ROS are continuously generated, and, depending upon the type and amount of species formed, the effects can be both deleterious and beneficial to the cell (Halliwell, 1992; Juurlink et al., 1998; Halliwell, 2001).

One of the most common ROS formed during normal mitochondrial respiration is a diatomic oxygen molecule containing an unpaired electron known as superoxide anion ($O_2^\cdot$). Outside of electron transport, superoxide can be produced through a number of ways, including arachidonic acid metabolism, phagocytic generation (monocytes, neutrophils, eosinophils, macrophages), and autooxidation of cysteine and catecholamines (Juurlink et al., 1998).
Figure 3. The production of reactive oxygen species.
Once formed, superoxide has the ability to react with cellular constituents (particularly proteins containing thiol groups and membrane lipids), as well as with other radicals such as NO•. The consequences of superoxide formation are often destructive, a desirable attribute when used by phagocytes to defend healthy cells from foreign organisms. However, not all pools of superoxide are damaging. Cell signaling processes and growth regulation are mediated by the release of superoxide from a variety of cell types (Halliwell, 1992). A scavenging mechanism exists to limit cellular exposure to superoxide anion whereby a superoxide dismutase (SOD) enzyme (Cu, Zn-SOD, Mn-SOD) converts superoxide to hydrogen peroxide and molecular oxygen.

Superoxide dismutation is the primary source of hydrogen peroxide (H₂O₂). Because H₂O₂ contains no unpaired electrons, it does not qualify as a free radical (Halliwell, 1992). The potential destructive nature of H₂O₂ lies in its ability to cross cell membranes easily and react with the transition metals, like iron. For example, through the Fenton reaction, H₂O₂ oxidizes iron (II) ion (Fe²⁺) to iron (III) ion (Fe³⁺) and the hydroxyl radical (•OH) and hydroxy ion are formed. The effects of hydroxyl radical formation are often severe. Hydroxyl radicals react with most macromolecules through the abstraction of an electron, inducing DNA strand breakage at the deoxyribose end of the nitrogenous bases, lipid peroxidation by abstracting a hydrogen atom from polyunsaturated fatty acid side chains, and enzyme inactivation through oxidation of protein thiol groups (often involving enzymes such as GSH peroxidase and GSH reductase that serve to buffer intracellular oxidants) (Juurlink et al., 1998; Halliwell, 2001).

Nitric oxide (NO•) is yet another deleterious mediator of oxidative stress. Nitric oxide reacts with O₂• to yield ONOO•, which can deliver a direct oxidative insult or can
decompose to form the more harmful •OH (Radi et al., 1991). Additionally, NO• inhibits cytochrome oxidase (complex IV) activity, thereby limiting oxygen consumption in respiring cells.

**THE CNS IS SUSCEPTIBLE TO OXIDATIVE DAMAGE**

Two main features of the CNS make it especially vulnerable to oxidative damage; (1) the capacity for reactive oxygen species (ROS) production is great, owing in part to the high metabolic demands of the CNS and (2) the neuronal specialization present in the CNS is membrane based, the primary components of which (lipids and proteins) are targets of oxidative damage.

The ATP requirements of the CNS are high and can be attributed to the establishment of membrane potentials, storage and release of neurotransmitter, and the synthesis of metabolites for normal function. The particularly high-energy needs of the CNS are coupled to 20% of the basal O2 consumption and thus incomplete mitochondrial reduction of O2 can be a significant source of ROS such as superoxide (for review see Halliwell, 1992). Other sources of ROS in the CNS, such as the autooxidation of dopamine and microglial activity (producing H2O2 and superoxide), compound the stress generated through aerobic respiration. The pathological threat posed by the presence of peroxides is heightened by the transition metal enrichment of the CNS where the reaction with iron may lead to •OH production. In conditions where brain injury occurs, especially in developing brains, the presence of neuronal NOS is known to play a major role in advancing the damage initiated by hypoxia (Black et al., 1995; Ferriero et al., 1996; Muramatsu et al., 2000). The heightened risk for ROS damage is furthered by an
unfavorable antioxidant environment. While ascorbate is generally perceived as a prominent antioxidant, the high concentrations of ascorbate in the gray and white matter of the CNS can have a pro-oxidant function (dependent upon the presence of iron or copper). Additionally, the activities of certain antioxidants (catalase) are low in the CNS and may contribute to higher than normal ROS damage.

Nervous system function is highly membrane based. The proximity of numerous neuronal membranes coupled with the potential for synaptic diffusion of transmitters requires selective regulation at the membrane level. The all-or-nothing generation of action potentials is a function unique to neurons and by design relies on proper membrane depolarization. Membrane ion channel proteins mediate depolarization and hyperpolarization primarily through passive and gated transport of Na⁺ and K⁺. Pre- and post-synaptic membrane receptors regulate the type (excitatory or inhibitory) and intensity of signal that is received by post-synaptic neurons following transmitter release. Enzymatic and transport processes that serve to terminate the signal are also mediated at the membrane level.Synaptic transmission, from the establishment of an action potential to termination of the signal is highly dependent on membrane function and integrity.

The lipid and protein components of the cellular membranes are primarily responsible for maintaining membrane integrity and mediating cellular function. Cells of the CNS have a distinct lipid composition that is rich in polyunsaturated fatty acids (docosahexaenoic acid C22:6), which are susceptible to radical attack leading to a destructive cascade of lipid peroxidation reactions. Lipid peroxidation is one of the most damaging consequences of hydroxyl radical production in the CNS (for reviews see Juurlink et al., 1998; Halliwell, 2001). The first step in lipid peroxidation is abstraction of
a hydrogen atom from a methylene carbon by hydroxyl radical. A lipid radical results, which gives rise to a peroxyl radical when combined with oxygen. The peroxyl radical is converted to a lipid hydroperoxide by abstracting a hydrogen atom from an adjacent polyunsaturated fatty acid and the cycle continues.

Lipid peroxides have the capacity to directly compromise glutamate transport through oxidation and structure alteration of the glutamate transporters. However, due to the non-specific and indiscriminate nature of most ROS, a change in glutamate transport may not imply that the oxidative insult is acting only at the site of the glutamate transporter. Upon exposure to ROS, other membrane proteins (ATPases, ion channels, receptors) essential for the energy and membrane potential requirements of glutamate neurotransmission, could indirectly alter glutamate transport. Therefore, understanding the vulnerability of the transporters to ROS damage is necessary to begin deciphering mechanisms of glutamatergic dysfunction.

The EAATs may be especially susceptible membrane proteins. It is believed that the transport activities of GLT1 (EAAT2), GLAST (EAAT1) and EAAC1 (EAAT3) are equally inhibited by oxidants through direct modification of the protein (Trotti et al., 1998). Commonly, targets of oxidation on proteins are cysteine sulfhydryl groups (Radi, 1991 #125) and the EAATs contain varied amounts of cysteine residues, two of which are conserved among all the subtypes, except for EAAT5, which has only one conserved cysteine (Kanner et al., 1993; Arriza et al., 1994; Fairman et al., 1995). Electrophysiological studies demonstrate that glutamate transport is inhibited and current flux decreased with the oxidation of sulfhydryl groups by 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB), and that the thiol-reducing agent dithiothreitol (DTT) reverses the
inhibition and restores the current flux (Trotti et al., 1997). This finding demonstrates that the chemical redox state of reactive cysteine residues may serve to regulate glutamate uptake. Direct application of oxidants H$_2$O$_2$ and xanthine/xanthine oxidase (X/XO) brought about a marked decrease (20% and 40% inhibition) in glutamate uptake in cultured astrocytes with no significant parallel release of lactate dehydrogenase. This suggests that glial cell viability is not jeopardized when glutamate transport is inhibited by ROS. Inhibition was prevented and partially restored with thiol reducing agents (DTT and GSH) but could not be restored or prevented with lipophilic antioxidants nor were any lipid peroxidation products present (Volterra et al., 1994). These findings indicate that in response to oxidizing agents, glutamate transport inhibition occurs independently from changes in cell viability or lipid alteration, and is primarily attributed to cellular changes occurring at the protein level.

**DEFENSES AGAINST THE THREAT OF ROS IN THE CNS**

The presence of ROS is compensated by the cell through endogenous antioxidant defenses. It is when the defenses of the cell are overcome by the generation of oxygen-derived species that oxidative stress exists (Halliwell, 1992). Under normal physiological conditions, electron extraction by unstable free radicals and their precursors is primarily prevented by both enzymatic and non-enzymatic detoxification systems. This is not to say that some level of ROS activity occurs and may even be required for cellular communication and regulation. Important enzymatic antioxidants include glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). GPX and CAT catalyze the reduction of hydrogen peroxide to water and molecular oxygen. The SOD
enzymes convert superoxide to hydrogen peroxide and water. Ascorbate, α-tocopherol, and glutathione are examples of non-enzymatic molecules that form stable intermediates with free radicals and terminate free radical chain reactions and limit macromolecule damage (for review see Halliwell, 2001). Ascorbate is water-soluble and serves to detoxify free radicals such as hydroxy radical and superoxide. α-Tocopherol is a lipid-soluble antioxidant and primarily protects against deleterious lipid peroxidation cycle in cellular membranes. Glutathione (GSH) is a major cellular thiol that is afforded substantial reducing power through its sulfhydryl-group containing cysteine residue. GSH is unique in that it participates in both enzymatic and non-enzymatic antioxidant processes (i.e. GSH can scavenge H2O2 through the action of glutathione peroxidase (GPx) and act as a direct thiol reducing agent)(Orlowski et al., 1976; Meister et al., 1983).

Glutathione is present in virtually all cell types and is the most prevalent antioxidant in the mammalian central nervous system. The precursor amino acids of glutathione (cysteine, glutamate, and glycine) react to form GSH in two ATP-dependent synthesis steps (Figure 4). GSH has been found to comprise 97% of the acid-soluble thiols in the cerebral cortex and approximately 80% of the total soluble thiol groups in the cerebral cortex. In whole brain, the concentration of total glutathione, consisting of both its oxidized and reduced forms, is 0.5-3.4 μmoles/gm tissue (Orlowski et al., 1976). Histochemical and biochemical methods reveal that GSH is localized primarily in astrocytes(Slivka et al., 1987; Sagara et al., 1993; Ben-Yoseph et al., 1996), which consequently can withstand higher concentrations of H2O2 induced toxicity than neurons (Raps et al., 1989; Philbert et al., 1991; Desagher, 1996). Increasing evidence suggests
that cells with depleted levels of glutathione are more vulnerable to the oxidative products of normal respiration, implicating glutathione as the primary antioxidant responsible for maintaining redox homeostasis (Murphy et al., 1990; Ben-Yoseph et al., 1996; Halliwell, 2001). In a pathological setting, it has been hypothesized that one link between dopaminergic cell death in the substantia nigra and the concomitant decrease in glutathione observed in patients with Parkinson’s disease may be the accumulation of oxidants.

Maintaining intracellular GSH levels is largely dependent upon the availability of the rate-limiting precursor cysteine (Orlowski et al., 1976; Bannai, 1986). Cystine, transported through the \( \text{Na}^+ \)-independent system \( X_c^- \), is reduced intracellularly and thought to be the primary source of cysteine for GSH synthesis in astrocytes and glioma cell lines (Cho et al., 1990; Sagara et al., 1993). The intracellular glutamate pool (maintained by glutamate uptake through the EAATs and glutamine deamination following glutamine uptake) is responsible for charging cystine uptake and maintaining intracellular GSH levels. Rimaniol and colleagues demonstrated that adding glutamate to the culture media increases intracellular GSH in a dose-dependent manner as long as sufficient cystine was present. The increase in GSH was not observed when glutamate was added in the presence of an EAAT inhibitor. Likewise, consistent with the competitive interaction of system \( X_c^- \) substrates, when the glutamate gradient is essentially reversed and the extracellular glutamate concentration is 1-10mM, cystine uptake is inhibited and GSH levels drop (Murphy et al., 1990; Sagara et al., 1993; Rimaniol et al., 2001). These results establish that glutamate transport through the EAATs maintain GSH levels by providing precursor molecule and establishing
Figure 4. The metabolism of glutathione. The rate-limiting reaction for GSH synthesis is catalyzed by gamma-glutamylcysteine synthetase (1). γ-Glutamylcysteine and glycine then react to form the final product in the second synthesis step, which is catalyzed by GSH synthetase (2). As GSH scavenges oxidants and free radicals it is converted to the oxidized form GSSG through the action of GSH peroxidase (3). Regeneration of GSH from GSSG requires the reducing cofactor NADPH and the enzyme GSH reductase (4). (Orlowski et al., 1976; Meister, 1991; Meister, 1995).
a glutamate pool allowing for the exchange of extracellular cystine for intracellular glutamate through system $X_c$ (Igo Jr. et al., 1998; Rimaniol et al., 2001).

Maintaining GSH levels in astrocytes is not only important for their own viability, but also for the protection of neurons, both directly with GSH and indirectly by providing neurons with precursors following the extracellular catabolism of GSH (Sagara et al., 1993; Schipper, 1996; Drukarch et al., 1997). Even though neurons are thought to exhibit cystine transport activity (Murphy et al., 1990; Sagara et al., 1993; Sagara et al., 1993) it is not likely to be through system $X_c$ as the neuronal uptake (Murphy et al., 1990) appears to exhibit different pharmacology than that in astrocytes (Cho et al., 1990). Additionally, northern blotting (Sato et al., 2002), and substrate immunoreactivity studies (Pow, 2001) indicate that system $X_c$ is predominantly expressed in glia. Neurons are thought to obtain the rate-limiting GSH precursor cysteine through direct uptake mechanisms (Zerangue et al., 1996; Chen et al., 2003) that when inhibited lead to a decrease in GSH synthesis (Chen et al., 2003). What is most important, with respect to GSH, is that independent of the neuronal or glial mechanism of cystine uptake, when the availability of intracellular cysteine is limited, GSH levels are attenuated.

Among the cellular antioxidant systems in the CNS, GSH is the most intimately connected with glutamate uptake. Ultimately, lowering the levels of glutathione in cells increases their sensitivity to excitotoxicity and oxidative stress (Bridges et al., 1991; Ben-Yoseph et al., 1996) and neurodegeneration is often a consequence (Ben-Yoseph et al., 1996; Schulz et al., 2000)
GLUTAMATE TRANSPORT AND A CYCLE OF EXCITOTOXICITY AND ROS FORMATION

The progression of many CNS pathologies, both acute neurological insults such as ischemia, trauma, and hypoglycemia as well as the chronic disease states such as Huntington’s, ALS, Alzheimer’s, and aging, may be a direct consequence of excitotoxicity and oxidative stress. One concept currently gaining acceptance is that excitotoxicity and oxidative damage may not be independent phenomena (for review see Trotti et al., 1998). Studies have shown that ROS are capable of inhibiting glutamate uptake directly (Piani et al., 1993; Volterra et al., 1994; Trotti et al., 1997; Rao et al., 2003). Because glutamate transport is responsible for regulating extracellular glutamate levels, a loss in transport activity can lead to excitotoxicity. Therefore, glutamate transport activity serves as a possible link between the potentially pathogenic events of oxidative stress and excitotoxicity.

Interestingly, it is now thought that one consequence of excitotoxicity is the production of ROS. As discussed earlier, excess glutamate levels are widely accepted to initiate excitotoxicity by stimulating NMDA receptors leading to inordinate Ca\(^{2+}\) influx (Choi, 1987; Reynolds et al., 1995). Calcium is thought to induce ROS production through a number of cellular targets, the combination of which most probably contributes to the observed presence of ROS in excitotoxic episodes (Lipton et al., 1994; Choi, 1995; Juurlink et al., 1998; Halliwell, 2001). In mitochondria, Ca\(^{2+}\) accumulation disrupts the mitochondrial membrane potential (Schinder et al., 1996) and has been shown to uncouple electron transfer from ATP synthesis leading to free radical generation (Dugan et al., 1995; Reynolds et al., 1995; Schinder et al., 1996). Another important source of
ROS attributed to NMDA-mediated Ca\(^{2+}\) influx is phospholipase A\(_2\), which is thought to be activated by Ca\(^{2+}\) and cause the release of arachidonic acid (Lazarewicz et al., 1990; Manzoni et al., 1997), the metabolism of which can be a source of ROS. Calcium also leads to the activation of xanthine oxidase, the enzyme responsible for the oxidation of hypoxanthine to xanthine and then uric acid, simultaneously producing O\(_2^-\) and H\(_2\)O\(_2\).

The synthesis of NO\(^•\) in the CNS is through a constitutive Ca\(^{2+}\)/calmodulin-dependent form of NOS (Marletta, 1994). Activation of nNOS can lead to glutamate release from synaptosomes (McNaught et al., 1998), OONO formation (for review see Juurlink et al., 1998), and •OH formation (Lancelot et al., 1998). Therefore, if NMDA activated Ca\(^{2+}\) influx resulting from excitotoxic levels of glutamate can produce ROS (Dugan et al., 1995; Reynolds et al., 1995; Rao et al., 2003), and ROS can inhibit glutamate transport, then transport activity does not merely link independent events of oxidative stress and excitotoxicity, but mediates the development of a potentially destructive cycle (Figure 5).

The existence of an excitotoxic cycle is sometimes best illustrated when examining acute neuronal pathologies such as trauma and ischemia. In traumatic neuronal injury, high levels of glutamate are thought to arise at an injury site due to damaged neurons, thereby initiating excitotoxicity in surrounding neurons. Soon after injury, inflammatory mediators and microglia, specialized macrophages of the CNS, are capable of releasing glutamate and free radicals, expanding the range of injury (Piani et al., 1993). Over-activation of glutamate ionotropic receptors and subsequent Na\(^+\) and Ca\(^{2+}\) entry may also lead to the production of ROS, possibly impairing glutamate transport by interacting with the transporters themselves, or other membrane proteins and lipids, both escalating and expanding the scope of injury by further elevating glutamate levels. Additionally,
Na$^+$ and Ca$^{2+}$ influx may lead to a depletion of ATP as ATPases attempt to restore ionic balances. At this point of energy deficiency, the pathology of trauma and ischemia overlap. In situations of ischemia, a disruption of blood flow causes a reduction in O$_2$, and ATP shortage often results. Whatever the cause, glutamate levels are also known to increase dramatically during energy failure (Choi et al., 1990; Lipton et al., 1994; Juurlink et al., 1998; McNaught et al., 1998; Zeevalk et al., 1998). The transporters derive their energy to accumulate glutamate intracellularly from ion gradients that ultimately are set up by the Na$^+$/K$^+$ ATPase. Under ischemic conditions, Na$^+$/K$^+$ ATPases are unable to function, Na$^+$ permeability is disrupted and membrane potentials are reduced. As Na$^+$ begins to increase inside cells, glutamate is released for at least three reasons (1) the Na$^+$-gradient is no longer able to drive and maintain the glutamate gradient and thus intracellular glutamate can travel through the transporters in reverse, (2) Na$^+$ influx will depolarize the cells allowing for increased synaptic release of glutamate, and (3) cell-swelling- induced release of glutamate may also occur as a result of the diminished Na$^+$/K$^+$ ATPase activity (Lipton et al., 1994; Takahashi et al., 1997; Seki et al., 1999). Clearly, the consequences of increased glutamate levels is eventually even higher glutamate levels, a self-perpetuating and self-destroying excitotoxic cycle.

In addition to acute pathologies such as ischemia, the co-dependence of excitotoxicity and ROS production for the establishment of chronic neurodegenerative diseases such as ALS, (Rao et al., 2003) Huntington’s chorea, (McGeer et al., 1978) Alzheimer’s disease (Ying, 1997), and aging (Ying, 1997) is also gaining acceptance (Lipton et al., 1994; Halliwell, 2001) and has become the premise for which therapeutic interventions are based (Juurlink et al., 1998; Halliwell, 2001).
Figure 5. Glutamate transport and a cycle of excitotoxicity and ROS formation
Specific Aims

RATIONALE

The present study was undertaken to examine the sensitivity of glutamate transport to a mild oxidative insult produced by glutathione depletion. The production of reactive oxygen species (ROS) is one mechanism that may adversely affect glutamate transport. Excessive oxidative stress has been shown to inhibit the glutamate transporters (Piani et al., 1993; Volterra et al., 1994; Trotti et al., 1997; Rao et al., 2003). Following inhibition of the EAATs, extracellular glutamate levels increase and can accumulate to excitotoxic levels, which, in time, can lead to excessive ROS production. The result of glutamate transport inhibition and ROS production can often be more than additive and propagation of one another can create a destructive cycle. Understanding the sensitivity of the transporters to ROS-mediated damage is necessary for deciphering excitotoxic and possibly pathogenic events involving glutamate neurotransmission.

A common approach to study the relationship between excitotoxicity and oxidative damage has been to introduce oxidants at levels high enough to induce excitotoxicity and death. Allowing cells to exist in an environment in which GSH levels are lowered provides a new, and possibly, more relevant way to study the link between excitotoxicity and oxidative stress with the primary focus being the vulnerability of glutamate transport.
EXPERIMENTAL APPROACH

AIM 1: Characterize the Na\(^+\)-dependent uptake activity in primary astrocytes and assay for intracellular glutathione.

**Hypothesis:** Long-term cultures of primary astrocytes exhibit stable levels of Na\(^+\)-dependent glutamate transport activity and intracellular glutathione.

**Rationale:** As glia are thought to be responsible for the majority of glutamate transport and glutathione synthesis, evidence of these processes should exist in primary astrocyte cultures.

**Specific Experiments**

1. Determine the subtype of glutamate transporter present in primary astrocytes.
2. Examine the pharmacological activity of the glutamate transporter expressed in primary astrocytes.
3. Establish the rates of \(^3\)H-D-aspartate uptake through the glutamate transporters in primary astrocytes over a 28-day time course.
4. Use the glutathione recycling assay to monitor total glutathione levels.

AIM 2: Assess the sensitivity of \(^3\)H-D-aspartate uptake activity in astrocytes chronically treated with an inhibitor of glutathione synthesis, buthionine sulfoximine (BSO)

**Hypothesis:** Glutamate transport activity is sensitive to endogenous oxidative stress produced by the chronic depletion of glutathione.

**Rationale:** The transport process has been shown to be sensitive to oxidative stress. Endogenous levels of ROS produced as a consequence of normal cellular metabolism may normally be scavenged by glutathione. The depletion of glutathione by BSO may cause oxidative stress sufficient to attenuate transport activity.

**Specific Experiments**

1. Quantify glutathione levels in primary astrocytes
2. Quantify glutathione levels in astrocytes chronically treated with BSO
3. Establish the rates of \(^3\)H-D-aspartate uptake through the glutamate transporters in primary astrocytes chronically treated with BSO
AIM 3. Assess the sensitivity of $^3$H-D-aspartate uptake activity in astrocytes chronically depleted of glutathione to an acute challenge of mild oxidative stress.

**Hypothesis:** Glutamate transport activity in astrocytes with depleted levels of intracellular glutathione may be more sensitive to a mild exogenous oxidant exposure.

**Rationale:** High levels of exogenously applied oxidants have been shown to inhibit transport. The attenuation of transport by sub-toxic levels of oxidants may be increased in astrocytes with depleted glutathione levels.

**Specific Experiments**

1. Apply moderate concentrations of $\text{H}_2\text{O}_2$ and X/XO to astrocytes

2. Apply moderate concentrations of $\text{H}_2\text{O}_2$ and X/XO to BSO-treated astrocytes

3. Establish the rates of $^3$H-D-aspartate uptake through the glutamate transporters in astrocytes receiving the acute challenge

AIM 4. Assess the levels of oxidative stress in astrocytes by measuring secondary markers of ROS and oxidative stress.

**Hypothesis:** As a result of oxidative stress produced by glutathione depletion and the added ROS, a change in tryptophan fluorescence should be observed that is indicative of protein/ROS interactions.

**Rationale:** ROS are often too labile to measure directly. A known marker of oxidative stress in proteins is the oxidation of tryptophan residues in proteins. This oxidation is associated with a measurable decrease in fluorescence. Protein oxidation is thought to be more sensitive than other techniques and is especially relevant to transporter inactivation. Using this technique, the effects of BSO and oxidants can be examined.

**Specific Experiments**

1. Assess the levels of protein oxidation in astrocytes treated with BSO using tryptophan fluorescence.

2. Assess levels of protein oxidation associated with acutely challenged astrocytes using tryptophan fluorescence.

3. Assess levels of protein oxidation in both BSO-treated and acutely challenged astrocytes using tryptophan fluorescence
Materials and Methods

**Primary Astrocyte Culture**

DMEM/F-12, (GibcoBRL CAT# 12400-024); 0.22 um membrane filter, (Fischer CAT#09-761-50); N-2-Hydroxyethylpiperazine-n-2-ethanesulfonic acid (HEPES), (Sigma H9136); Bovine serum albumin, (Sigma A7888); Hank's Balanced Salt Solution, (HBSS) (Gibco 310-4180 AG); Penicillin/Streptomycin, (Gibco 600-5140 PE); Fungizone, (Gibco 600-529 AE); Fetal Bovine Serum, (heat inactivated) (Gibco 230-6140 AJ); Trypsin, (Gibco 610-5055 AG); sodium pyruvate, sodium bicarbonate, and EDTA were purchased from Sigma. 12 well Corning Costar polystyrene plates, (Fisher 0720082); 150 cm² cell culture flasks, (Fisher 430824); L-buthionine-[S,R]- sulfoximine (BSO), (Sigma B-2515)

Primary astrocytes were prepared based on a standard protocol (McCarthy et al., 1980). In brief, three 3-4 day-old Sprague-Dawley rat pups were sacrificed and the brains placed into a 60 mm dish containing Ca²⁺/Mg²⁺ free buffer (CMF) on the stage of a dissecting microscope. The meninges were removed, brain divided into hemispheres, and the midbrain and hippocampus dissected out. The remaining cerebral cortices were washed in CMF and triturated in a 15 ml centrifuge tube with a 10 ml pipette. Tissue was incubated for 10 min in 37°C water bath and centrifuged in clinical centrifuge for 3 minutes. The pellet was suspended in 6 mls DMEM / F12 W / 15% FBS, triturated with a flame-constricted siliconized glass pipette, and suspension was evenly distributed (2ml) into three 150 cm² flasks containing 30 mls DMEM / F12 W / 15% FBS. Following a 24 hr
incubation at 37° C, cells were rinsed with 10mls DMEM/ F12 and fed with 30 mls of DMEM/ F12 w/ 10% FBS. Every 4 days, cells were fed with 30 mls of fresh DMEM/ F12 w/ 10% FBS. On day 8, flasks were shaken at 280 rpm for 24 hours, rinsed with DMEM/ F12, fed with 30 mls of DMEM/ F12 w/ 10% FBS, and allowed to incubate at 37° C for five days. Following purification, the 14 day old primary astrocyte culture cells were harvested with 0.25 % Trypsin solution, transferred to 50 ml tubes, centrifuged for 5 min, pellet suspended in DMEM/ F12 w/ 10%FBS, trituated with a flame-constricted siliconized glass pipette, and counted. Cells were then plated on 12-well plates at a density of ~ 25,000 cells/well. Seven to eight days post-plating astrocytes reach confluency and were designated as experimental day 0. Feeding took place every 4 days up to 28 days with DMEM/ F12 w/ 10% FBS for controls and DMEM/ F12 w/ 10% FBS/500 μM BSO in treated wells. D-Aspartate uptake assays were performed on experimental days 1, 7, 14, and 28 post-plating (22, 29, 36, 50 days in culture respectively) depending upon experimental conditions (days 1 and 7 only for H₂O₂ and X/XO insult).

**Glutathione Recycling Assay**

Sulfosalicylic acid, (Sigma S-2130); Glutathione, (Sigma G-6529); NADPH, (Sigma N-1630); Glutathione reductase, (Roche 105-678); DTNB, (Sigma D-8130)

Quantification of total GSH was achieved using an enzymatic-recycling assay described by Anderson, 1985 (Anderson, 1985). 5, 5’-dithiobis-2-nitrobenzoic acid (DTNB) serves to oxidize GSH to GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid
GSSG is reduced to GSH by GSH reductase and NADPH. The rate of TNB production was monitored at 405 nm for 5 minutes on a Molecular Devices ThermoMax Microplate spectrophotometer and represents total glutathione content. On day of assay primary astrocytes were harvested at 20-48 days old (1-28 days post-plating), homogenized in 1% sulfosalicylic acid (SSA), microfuged for 1 min, and kept at 4°C until use. The assay was performed by combining 50 μl each of DTNB, NADPH, GSH reductase, and sample (supernatant) in a 96-well microplate. The final concentrations were as follows: 0.6 mM DTNB, 0.2 mM NADPH, and 0.66 U/ml GSH reductase.

**3H-D-Aspartate Transport Assays**

Assay buffer (1 liter): CaCl₂ (1.1mM), MgSO₄·7H₂O (0.71 mM), D-Glucose (5.5mM), HEPES (10mM), HBSS: KCl (0.4 g/L), KH₂PO₄ (0.06 g/L), NaCl (8.0 g/L), Na₂HPO₄·7H₂O (0.09 g/L), D-Glucose (1.0 g/L). Xanthine (X) Sigma X-0626; xanthine oxidase (XO) (Sigma X-4500); H₂O₂, (Mallinckrodt AR 5240); Dimethyl sulphoxide DMSO (Sigma D-2650); D-aspartate (Sigma A-8881); ³H-D-aspartate obtained in 1.0 mCi vials, S.A. 15.60 Ci/mmol from Perkin Elmer Life Sciences Inc.; SDS, (Bio-Rad 161-0416).

On the day of the assay, untreated astrocytes and astrocytes treated with BSO were removed from 37°C incubation, media was removed, replaced with assay buffer and either assayed for D-aspartate uptake (in the presence or absence of DHK 250μM FV and BSO 500μM FV) or insulted first with X/XO (500μM/5mU/ml FV) or H₂O₂ (50μM FV), incubated at 37°C for 15 minutes, and subsequently assayed. The D-aspartate uptake
The protocol in short is as follows: 12-well Costar plates containing primary astrocytes were incubated for 5 minutes in HBSS control buffer pH 7.4 in water bath at 30°C. Control buffer was aspirated and FV 25 μM ³H-D-Aspartate was aliquoted to each well. Following a 5-minute incubation, uptake was immediately stopped through aspiration of substrate and quenching with ice-cold control buffer. 0.4N NaOH was added to each well and allowed to shake slowly for 24 hrs. 200 µl aliquots were then transferred to vials with 4-6 µl glacial acetic acid and 3.5 mls Liquiscint. The rate of ³H-D-aspartate uptake was quantified through liquid scintillation counting, corrected for non-specific uptake at 4°C, and normalized for protein using the BCA method.

**IMMUNOBLOTTING**

Complete lysis buffer, (Roche); 10% Tris-HCl gel, (Bio-Rad); Hybond-P PVDF membrane, (Amersham Pharmacia Biotech); anti-rat GLAST, (Alpha Diagnostic International); rGLT-1, (Affinity BioReagents); donkey anti-rabbit IgG-F(ab’)2-HRP, (Amersham Biosciences); anti-biotin, (Cell Signaling); ECL reagents, (Amersham Biosciences); Versadoc Imaging System, (Bio-Rad).

Cultured astrocytes grown in 150 cm² dishes were washed and harvested on days 20 and 34 (day 1 and 14 post-plating) in cold PBS. Cell suspension was spun at 1000-1200 RPM (#3) for 10 minutes, the supernatant was removed and cell pellet was immediately put on ice. Pre-chilled Complete lysis buffer was added to pellet (0.25 mls/dish) and cells were homogenized on ice with a 27-gauge needle. Protein was quantified using the Bradford assay and stored at −20°C until use. On the day of the immunoblot, samples were thawed
on ice and denatured by boiling for 5 minutes in 1X Laemmli buffer. Proteins were separated using SDS-polyacrylamide gel electrophoresis in Tris/glycine/SDS buffer between 120-150 volts on a 10% Tris-HCl gel. Proteins were transferred to a Hybond-P PVDF membrane in tris/glycine/20% methanol pH 8.3. Molecular weight standards were obtained from Cell Signaling. GLAST blots were blocked in 0.1% skim milk/tris-buffered saline (20mM Tris Base/130mM NaCl) 0.1% Tween (pH 7.6) for 1 hour and then probed with affinity purified rabbit anti-rat GLAST against a 15 aa synthetic peptide near the C-terminus of rGLAST at 1:250 at 4°C overnight. GLT-1 blots were blocked in 0.5% skim milk/tris-buffered saline (20mM Tris Base/130mM NaCl) 0.1% Tween (pH 7.6) for 1 hour and then probed with rabbit anti rGLT-1 antiserum against a 17 aa synthetic peptide at 1:1000 at 4°C overnight. Following probing, blots were incubated with donkey anti-rabbit IgG-F(ab')2-HRP (Amersham Biosciences) at 1:5000 and antbiotin at 1:1000 in 0.1% or 0.5% milk TBS/Tween for 1 hour at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) on the Versadoc Imaging System.

**TRYPTOPHAN FLUORESCENCE**

Tryptophan residues account for the majority of protein fluorescence. A microplate fluorometer using Softmax Pro software was used for relative fluorescence readings in astrocytes (treated and untreated) as follows: On day of harvesting, DMEM/F12 w/ 10% FBS was removed from astrocytes, washed with HBSS 2x, and scraped in 1% SSA when looking at the effect of BSO over the 28 day course. For cells to be insulted, assay buffer was added following the wash. Astrocytes (BSO treated or un-treated) were then exposed
to either X/XO (500μM/5mU/ml FV) or H₂O₂ (50μM FV) at 20-27 days old (day 1 and 7 post-plating) and allowed to incubate at 37°C for 15 minutes as in uptake experiments. Following insult incubation, cells were harvested from 12-well tissue culture plates in 1% SSA and homogenized. Following protein determination, equivalent amounts of protein were centrifuged on a high-speed microfuge, resuspended in 1% SDS, re-homogenized, a maximum wavelength spectrum is run, and the relative fluorescence determined. Bovine serum albumin (BSA) experiments were performed in 100 μl of the indicated solvents at the given protein amounts and the relative fluorescence determined.
Results

Transporter function is credited with maintaining proper glutamate homeostasis at the synapse. Because the central goal of this work was to reveal the vulnerability of glutamate transport to oxidative inactivation, it was important to identify a cell culture system that expressed glutamate transporters. A primary culture of cortical astrocytes was utilized for this study. This culture system was chosen as glia are thought to account for the majority of glutamate uptake in the brain, expressing both GLAST and GLT-1 (Rothstein et al., 1994; Rothstein et al., 1996; Swanson et al., 1997). Additionally, astrocytes are a major source of glutathione synthesis in the CNS (Slivka et al., 1987; Raps et al., 1989; Philbert et al., 1991; Sagara et al., 1993), making them an appropriate choice for an antioxidant-depletion model of oxidative insult. As a primary culture, the astrocytes are also considered more relevant than an immortalized model.

Astrocytes Exhibit Typical Morphology and Primarily Express GLAST

Astrocytes were isolated from 3-4 day old rat pups, purified, and after 22 days in culture (experimental day 1), were visualized on an Olympus 1X70 microscope at 20x magnification and photographed through a Hoffman Modulation Contrast lens using Wasabi software. Essentially pure cultures of astrocytes (>98%) are obtained using this method (McCarthy et al., 1980). The primary cells appear to grow in flat sheets and exhibit the typical polygonal morphology of an undifferentiated neuron-free culture (Figure 6) (Swanson et al., 1997; Schlag et al., 1998).
Transporter expression in the primary astrocytes was confirmed through immunoblotting for the two transporter subtypes known to be expressed in glia (Rothstein et al., 1994). Anti-peptide antibodies were obtained for rGLT-1 (ABR) and rGLAST (Alpha Diagnostic) and used to examine the presence of GLAST and GLT-1 in the primary astrocyte cell lysate. Proteins were separated using SDS-polyacrylamide gel electrophoresis in Tris/glycine/SDS buffer between 120-150 volts on a 10% Tris-HCl gel (Bio-Rad). Rat cerebellum lysate was used as a positive control for both GLAST and GLT-1 blots. The expression of the GLAST subtype of glutamate transporter predominated in the primary astrocyte culture (Figure 7 and Figure 8). The anti-rGLAST antibody recognized a protein with a molecular weight of ~50 kDa in both day 1 and day 14 cultures. GLT-1 expression in astrocyte cultures was not detected. Lanes probed with anti-rGLT-1 reveal a ~90 kDa that is likely the result of nonspecific antisera binding. These results are consistent with findings cited in the literature (Rothstein et al., 1994; Swanson et al., 1997). Even though GLT-1 is a known glial glutamate transporter, when grown in isolation from neurons under normal culture conditions, astrocytes primarily express GLAST (Swanson et al., 1997; Schlag et al., 1998).

**D-Aspartate Uptake Activity in Primary Astrocytes**

Radiolabeled D-aspartate uptake was used to quantify the activity of GLAST in primary astrocytes. Kinetic studies have established both D-aspartate and L-aspartate as competitive inhibitors and substrates of both GLT- and GLAST (Bridges et al., 1999). $^3$H-D-aspartate was chosen for uptake studies because it serves as a non-metabolizable glutamate analogue that is not present endogenously in cells (Drejer et al., 1983). In
addition to the mean rate of uptake (see below), transport activity of GLAST was also determined in the presence of DHK (a selective inhibitor of GLT-1) (Arriza et al., 1994) and BSO (a glutamate analog that inhibits γ-glutamylcysteine synthetase, γGCS) (Meister, 1991). On experimental day 1 (22 days in culture) primary astrocytes were incubated with 25μM ³H-D-Aspartate and 250μM DHK or 500μM BSO for 5 minutes at 30°C. Nonspecific uptake was corrected for by subtracting accumulated ³H-D-aspartate at 4°C. The rate of D-aspartate uptake in control astrocytes was 189 +/- 17.3 (pmol/min/mg +/- SEM). Neither DHK (163 +/- 12.1) nor BSO (218 +/- 2.14) had a large effect on D-aspartate uptake (Table 1). As a selective inhibitor for EAAT2 (GLT-1) (Arriza et al., 1994) this data is consistent with a culture primarily expressing the GLAST subtype of glutamate transporters. The Kᵢ for DHK in mammalian cells expressing EAAT1 (GLAST) is in the millimolar range and in the micromolar range for EAAT2 (Bridges et al., 1999).

The inability of BSO to inhibit uptake is important for two reasons. (1) As a glutamate analog and inhibitor of γ-glutamylcysteine synthetase, it was important to demonstrate that its action did not involve inhibition of D-aspartate uptake at GLAST. (2) Although not directly related to this study, the result here indicates that the mechanism by which BSO gains access to the intracellular space is not through the glutamate transporter. Currently the mechanism of cellular entry by BSO is unknown. It is concluded from the pharmacology data and the western blots shown in Figure 7 and Figure 8 that the predominant source of D-aspartate uptake in the primary astrocyte culture is through GLAST.
Following the inhibition studies, uptake experiments were performed in the astrocytes as a function of time in culture. On the day of the assay, untreated astrocytes (no BSO) at 20-48 days old (1-28 days post-plating) were incubated with 25 μM $^3$H-D-Aspartate for 5 minutes at 30°C. Nonspecific uptake was corrected for by subtracting accumulated $^3$H-D-aspartate at 4°C. As reported in Table 2 uptake rates for controls on days 1, 7, 14, and 28 are 201 +/- 34.5, 359 +/- 39.1, 419 +/- 75.3, and 386 +/-26.0 respectively (pmol/min/mg +/- SEM).

Modest increases in uptake were observed after day 1 (Table 2), suggesting increased transporter expression in long-term cultures. This would not be surprising as trafficking of membrane proteins to the plasma membrane from an intracellular pool, (providing a highly efficient form of protein expression that may exist in lieu of and in conjunction with protein synthesis) is employed by many membrane proteins (GABA, dopamine, and serotonin transporters, GLUT4). Rapid changes in cell membrane expression of the glutamate transporters are known to occur, although the details are not completely elucidated (Robinson, 2002).

Protein levels throughout the time course remain consistent. Uptake rates were normalized for total cellular protein using the bicinchoninic acid assay and values of 0.189 +/- 0.03, 0.220 +/- 0.02, 0.233 +/- 0.012, and 0.266 +/- 0.029 (mg/ml +/- SEM) were obtained from the standard curve on days 1, 7, 14, and 28 respectively (Figure 9). The astrocytes have reached ~90% confluency on experimental day 0 and appear to maintain near maximal confluency throughout the time course.
GSH LEVELS IN PRIMARY ASTROCYTES ARE DEPLETED WITH BSO

To quantify GSH levels within astrocytes a GSH recycling assays was used. The method employed is a modification of that of Tietze (Anderson, 1985) whereby 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) serves to oxidize GSH to GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to GSH through the action of the GSH reductase and NADPH included in the assay. Following homogenization of cell samples, the rate of TNB production is monitored at 405 nm for 5 minutes. Individual rates of TNB formation are determined for each amount of GSH and are used to generate a standard curve. A typical plot of individual rates over a range of GSH levels is depicted in Figure 10.

Originally, the rate of TNB formation for each standard and sample was carried out in individual cuvettes and absorbance recorded. To modify the procedure to a more time efficient microplate assay, the volumes of all reaction reagents (GSH, GSH reductase, DTNB, NADPH) were adjusted to accommodate the 200 μl well volume on a 96-well microplate. The final volume concentrations were as follows: 0.6 mM DTNB, 0.2 mM NADPH, and 0.66 U/ml GSH reductase. The microplate GSH recycling assay was performed over a range of known amounts of GSH (0.04 to 2.5 nmol) to access the reliability of the procedure (Table 3). The percent recovery value for GSH was approximately 98% over the range of concentrations examined. Having established the reliability of the microplate assay, a compatibility study between the cuvette and microplate methods for the GSH recycling assays was performed with cell extracts. C6 glioma cells were used to obtain a baseline for GSH levels as well as in cells treated with known GSH-depleting agents 4-S-CPG, a compound that limits the availability of the
GSH precursor cysteine (Patel et al., 2004), and BSO, the inhibitor of the rate limiting step of GSH synthesis (γGCS) (Meister, 1991). Control values for nmols of GSH in both the cuvette and microplate samples were similar, 2.7 +/- 0.06 and 3.1 +/- 0.05 respectively (nmol +/- SEM). Cells receiving 100 µM 4-S-CPG or 500 µM BSO using both the cuvette method (0.23 +/- 0.01, and 0.19 +/- 0.01) and the microplate method (0.20 +/- 0.02, and 0.16 +/- 0.01) yielded similar results (nmol +/- SEM) (Table 4). The data for 4-S-CPG and BSO is consistent with their ability to limit GSH synthesis and no significant difference was found to exist between the two methods.

The microplate assay was then employed to measure the amount of GSH in primary astrocytes as described in Methods. As in the previous uptake experiments (Table 2) the GSH content in astrocytes was examined as a function of time in culture. On experimental days 1, 7, 14, and 28 (22, 29, 36, 50 days in culture respectively) untreated (no BSO) primary astrocytes were found to contain 9.59 +/- 0.769, 8.34 +/- 0.286, 6.56 +/- 0.910, and 8.24 +/- 2.042 (mean GSH in nmol/mg +/- SEM) (Figure 11).

As with transport rates, there appears to be no effect of culture age on GSH levels. To more completely investigate the relationship between transporter function and GSH levels, D-aspartate uptake was assayed under GSH-depleted conditions. To insure that GSH within astrocytes could be effectively depleted, GSH levels were monitored following treatment with BSO. BSO is an effective inhibitor of GSH synthesis and has been shown to be less toxic and more selective than other GSH depleting agents (Meister, 1991).

On experimental day 1, 500 µM BSO was introduced with normal growth media (DMEM/ F12w/ 10% FBS) to primary astrocytes incubated at 37°C. Over the next 24
hours samples were assayed for GSH using the GSH recycling assay as described in Methods. At time 0, 4, 8, 12, and 20 hours samples were found to contain 4.0 +/- 0.31, 2.3 +/- 0.09, 1.0 +/- 0.04, 0.7 +/- 0.4, and 0.3 +/- 0.09 respectively (mean GSH in nmol/mg +/- SEM) (Figure 12). This confirmed the findings of others that the time required for BSO to deplete total GSH levels was under 24 hours when using 500 μM of inhibitor (Bridges et al., 1991; Devesa et al., 1993; Drukarch et al., 1997). To then establish an astrocyte culture chronically depleted of GSH, treatment with 500 μM was carried out during routine media changes for 28 days and cells were harvested using 1% SSA. On experimental days 1, 7, 14, and 28 the GSH levels in the primary astrocytes were 1.64 +/- 0.292, 1.34 +/- 0.119, 0.91 +/- 0.248, and 0.99 +/- 0.424 (mean GSH in nmol/mg +/- SEM) (Figure 13). The difference between untreated and treated astrocytes over the time course amounts to a 6-7-fold decrease in intracellular GSH (Table 5). GSH content in BSO-treated cells expressed as the percent of control levels for days 1, 7, 14, and 28 were 17 +/- 1.8, 16 +/- 1.3, 13 +/- 2.2, and 11 +/- 3.2 (Table 5). As shown in untreated cells, the GSH values remain stable when treated with BSO over the time course.

It appears that there exists some level of residual GSH in the astrocyte culture (Table 5) as previously observed in other cells treated with BSO (Bridges et al., 1991; Devesa et al., 1993; Stokes et al., 2000). The inability to totally eliminate GSH levels may exist for a variety of reasons (Meister, 1991 for review). Because BSO inhibits the active site of γ-glutamylcysteine synthetase and binds to the same site as glutamate, it is possible that a high levels of endogenous glutamate could compete with BSO inhibition of the enzyme. Negligible amounts of glutamate in the cellular media reduce the
likelihood of this possibility. Alternatively, GSH levels can be independent of γ-glutamylcysteine synthetase activity. This could occur if certain γ-glutamyl amino acids produced by γ-glutamyltranspeptidase are transported into the cell. For example, when cystine is the amino acid acceptor in this reaction, γ-glutamylcystine is formed and is transhydrogenated to form γ-glutamylcysteine, which can bypass the rate-limiting step and allow for minimal synthesis to take place. In addition to bypassing normal synthesis, a distinct subcellular pool of GSH unaffected by γGCS inhibition exists in mitochondria and could account for the residual levels of GSH (Meister, 1991). Mitochondria do not contain γ-glutamylcysteine synthetase or GSH synthetase and thus BSO uptake is not relevant. A form of GSH transport is thought to exist instead and thus provides a way for mitochondria to detoxify the fraction of incompletely reduced oxygen during aerobic respiration (McNaught et al., 2000).

**ASTROCYTES CHRONICALLY DEPLETED OF GSH MAINTAIN NORMAL TRANSPORT ACTIVITY**

Once the glutamate transporter subtype and activity was characterized in the primary astrocytes, and a protocol for depletion of GSH was established, uptake experiments were performed to examine whether or not time in culture in combination with lowered GSH levels left the transporters vulnerable to endogenous oxidative damage. Astrocytes were treated with BSO during routine media changes and subsequently assayed on experimental days 1, 7, 14, and 28 post-plating (22, 29, 36, 50 days in culture respectively) for 3H -D-aspartate uptake. Nonspecific uptake was corrected for by subtracting accumulated 3H-D-aspartate at 4°C. Uptake rates for BSO-treated astrocytes
are 204 +/- 38.4, 334 +/- 41.2, 341 +/- 50.6, and 366 +/- 29.0 on days 1, 7, 14, and 28 respectively (pmol/min/mg +/- SEM) (Table 6). Uptake rates for astrocytes exposed to BSO do not differ significantly from controls as demonstrated by percent of control uptake +/- SEM for days 1, 7, 14, and 28 (i.e. 94 +/- 2.75, 90 +/- 2.11, 84 +/- 5.10, and 90 +/- 5.45, respectively) (Table 6).

These findings suggest that under the conditions examined, GLAST mediated uptake is insensitive to the endogenous oxidative stress that may occur as a result of depleted GSH levels. If the concentrations of GSH in cellular media is high, then there exists the possibility that masking of both extracellular effects of internally produced ROS and of externally applied ROS (which may have both intracellular and extracellular effects). To insure that additional GSH was not introduced exogenously, the cellular media was also assayed for GSH content. The GSH content in DMEM/F12 w/10% FBS was determined by spiking the media with known amounts of GSH. The GSH content was approximately 0.319 nmol/ml. This value is similar to the amount of GSH present in cells treated with BSO (using a mean protein value of 0.2268 mg/ml yields 0.277 nmol/ml). It is concluded that the GSH level in the cell media is insufficient to overcome the effects of BSO in the astrocyte culture.

A MODEST ACUTE INSULT OF H\textsubscript{2}O\textsubscript{2} AND X/XO ALTERS TRANSPORT ACTIVITY IN UNTREATED ASTROCYTE CULTURES

To verify the sensitivity of glutamate transport function to oxidative damage, astrocytes were acutely treated with H\textsubscript{2}O\textsubscript{2} and xanthine/xanthine oxidase (X/XO) at a concentration
known to be non-toxic as determined by the release of the intracellular enzyme LDH (Volterra et al., 1994; Webster, 1998).

On the day of the assay (day 1 and day 7) astrocytes were exposed to either 50 μM H₂O₂ or 500 μM/5mU/ml X/XO for 15 min at 37°C and immediately washed in AB and assayed for D-aspartate uptake as described in Methods. On days 1 and 7 uptake was reduced in the presence of H₂O₂ to 189 +/- 35.7 and 201 +/- 23.4 (pmol/min/mg +/- SEM). These values correspond to decreases of 81 +/- 5.338 and 71 +/- 6.346 (% of control uptake +/- SEM) (Table 7). Following X/XO exposure on day 1 and 7 uptake was decreased to 169 +/- 42.8 and 139 +/- 11.1 (pmol/min/mg +/- SEM), corresponding to percent of control values of 70 +/- 5.788 and 59 +/- 5.858 (% of control uptake +/- SEM) (Table 7). Higher doses of both H₂O₂ (500μM) and X/XO (500μM/50mU/ml) have been shown to decrease glutamate uptake in astrocytes without a significant parallel release in LDH (Volterra et al., 1994).

A MODEST ACUTE INSULT OF H₂O₂ AND X/XO ALTERS TRANSPORT ACTIVITY IN GSH-depleted Astrocyte Cultures

In a subsequent series of experiments the same insult was applied to cells depleted of GSH. Astrocytes were treated at regular intervals with BSO and incubated with H₂O₂ and X/XO as described in Methods. Table 8 indicates that for astrocytes treated with 50 μM H₂O₂ in the presence of 500 μM BSO on day 1 and day 7 the uptake was decreased to 141 +/- 29.0 and 142 +/- 12.4 (pmol/min/mg +/- SEM), corresponding to percent of control values of 67 +/- 11.7 and 75 +/- 5.78 (% of control uptake +/- SEM). For those cells treated with 500μM/5mU/ml X/XO, results are similar for days 1 and 7, with uptake
decreased to 130 +/- 8.7 and 111 +/- 8.4 (pmol/min/mg +/- SEM), corresponding to percent of control values of 58 +/- 4.58 and 67 +/- 3.06 (% of control uptake +/- SEM) (Table 8). When the data for astrocytes exposed to H₂O₂ and X/XO was compared to that for cells treated under the identical conditions, but with decreased GSH levels, no significant difference in the amount of D-aspartate uptake was observed (Figure 14).

**Experimental Conditions Are Insufficient to Demonstrate Protein Fluorescence Changes in Astrocyte Cultures**

The fluorescence of the aromatic moiety on tryptophan residues has been used as an indicator of protein integrity in cell samples. Protein fluorescence is typically excited at 280 nm, with emissions at 348 nm attributed to tryptophan residues along with tyrosine and phenylalanine. The fluorescence yield of tryptophan is much higher than the other two and has different lifetime in the excited state and explains why tryptophan is predominant in emission. A loss in fluorescence correlates with the oxidation of tryptophan (Davies et al., 1987; Jones et al., 1987; LeBel et al., 1991).

No detectable change in tryptophan fluorescence was observed in BSO-depleted astrocytes on experimental days 1 through 28. The choice of solvent was based on literature methods (Davies et al., 1987; LeBel et al., 1991) and pre-existing cell sample solutions available. BSO was administered with media changes (DMEM/F12 w/ 10% FBS) every fourth day in culture. Following protein determination, equivalent amounts of protein were centrifuged on a high-speed microfuge, resuspended in 1% SDS, re-homogenized, and relative fluorescence determined at ex 280λ em 345λ. The sensitivity of fluorescence to a protein range relevant to the cell homogenate (0.005mg-0.08mg) was
tested in 1% SDS with no appreciable difference seen within the range (data not shown).
On days 1, 7, 14, and 28 the fluorescence readings of BSO-treated astrocytes were 94.0 +/− 5.37, 93.0 +/− 3.74, 85.0 +/− 5.15, and 88.0 +/−5.77 (% of control RFU +/− SEM) (Figure 15). There were no indications that BSO treatment over 28 day time course was concurrent with protein damage as measured by tryptophan fluorescence.

As the methods indicate, the exposure of astrocytes to either H₂O₂ or X/XO occurs in assay buffer (AB) and was allowed to incubate for 15 minutes at 37°C. The impact of this oxidant exposure is seen in uptake experiments as a loss in activity. If protein oxidation is taking place, it should be measurable using tryptophan fluorescence unless interference by the AB, the precipitation buffer (1% SSA) or by the solvent in which fluorescence is recorded (1% SDS), exists. To examine the quenching ability of these solutions, the tryptophan fluorescence of bovine serum albumin (BSA) exposed to oxidatants was assayed in both AB as well as 1% SDS (did not use 1% SSA because it precipitates protein and made homogenous sample prep difficult). BSA experiments were performed in 100μl of the indicated solvents with 0.05 mg BSA and treated for 15 minutes with a range of H₂O₂ concentrations. Over the 15 minute time period it took at least 100 μM H₂O₂ to demonstrate significant changes in fluorescence values verses controls in both solvents (p = 0.0003 for AB and p = 0.003 for 1% SDS) (data not shown).

Untreated astrocytes (no BSO) were exposed to nontoxic levels of H₂O₂ and X/XO as described in Methods on days 1 and 7 and assayed for tryptophan fluorescence. These cells received the identical treatment as those from Table 7 where D-aspartate uptake values were lowered upon insult exposure. Fluorescence values for day 1 and 7
are as follows: Day 1 (control, 27251 +/- 1359; H$_2$O$_2$, 27607 +/- 1897; X/XO, 27255 +/- 1635, and 1% SDS, 669 +/- 96.5); Day 7 (control, 25222 +/- 1733; H$_2$O$_2$, 26351 +/- 1579; X/XO, 26736 +/- 1563) (RFU +/- SEM) and are depicted in Figure 16. Unaltered RFU values over the seven-day time course were observed with both 50 μM H$_2$O$_2$ and 500 μM/5mU/ml X/XO treatment.

Under conditions of both oxidative insult and BSO treatment, fluorescence values for day 1 and 7 astrocytes were recorded as described in Methods. These cells received the identical treatment as those from Table 8. The fluorescence values were as follows: Day 1 (control, 26638 +/- 1906; H$_2$O$_2$, 27707 +/- 1576; X/XO, 27351 +/- 1639, and 1% SDS, 669 +/- 96.5); Day 7 (control, 25627 +/- 1730; H$_2$O$_2$, 25763 +/- 1079; X/XO, 26184 +/- 1275) (RFU +/- SEM) and are depicted in Figure 17. The values for the blank, 1% SDS, were 669 +/- 96.5. Similar to the untreated astrocytes (Figure 16), tryptophan fluorescence assays for BSO-treated astrocytes indicate no significant protein oxidation. Fluorescence values for untreated and BSO-treated astrocytes under oxidative insult are summarized in Figure 18. Exposure of primary astrocytes to moderate levels of oxidative insult appears to have different effects on uptake (see summary Table 9) and fluorescence (see summary Figure 18) but result in similar changes within each method when comparing untreated and BSO-treated cells.
Figure 6. Day 1 primary astrocytes prepared from 3-4 day-old Sprague-Dawley rat pups. Images were visualized on an Olympus 1X70 microscope at 20x magnification and photographed through a Hoffman Modulation Contrast lens using Wasabi software.
Figure 7. Western analysis of day 1 primary astrocytes. Cell lysate from day 1 cultures was subjected to SDS-polyacrylamide gel electrophoresis at 120-150 volts on a 10% Tris-HCl gel and immunoblotted with antibodies to rGLAST (A) and rGLT-1 (B). Loading volumes (40 μl) and protein quantities were identical for both blots. Lane 1, MW standards; lane 2, cerebellum lysate (20μg); lane 3, astrocyte lysate (45μg).
Figure 8. Western analysis of day 14 primary astrocytes. Cell lysate from day 14 cultures was subjected to SDS-polyacrylamide gel electrophoresis at 120-150 volts on a 10% Tris-HCl gel and immunoblotted with antibodies to rGLAST (A) and rGLT-1 (B). Loading volumes (40 µl) and protein quantities were identical for both blots. Lane 1, MW standards; lane 2, cerebellum lysate (20 µg); lane 3, astrocyte lysate (45 µg).
Table 1. Pharmacological activity of $^3$H-D-aspartate uptake in primary astrocytes.
Uptake values in presence of 25 μM $^3$H-D-aspartate measured over 5 min at 30°C.
Shown are mean values that arise from duplicate readings from duplicate sample wells
+/- SEM values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Rate (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-aspartate</td>
<td>25 μM</td>
<td>189 +/- 17.3</td>
</tr>
<tr>
<td>Dihydrokainate</td>
<td>250 μM</td>
<td>163 +/- 12.1</td>
</tr>
<tr>
<td>Buthionine sulfoximine</td>
<td>500 μM</td>
<td>218 +/- 2.14</td>
</tr>
<tr>
<td>Time (experimental days)</td>
<td>Rate of uptake (pmol/min/mg)</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Total (30°C)</td>
<td>Non-specific (4°C)</td>
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<td>1</td>
<td>218 +/- 35.9</td>
<td>17 +/- 4.3</td>
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<td>7</td>
<td>377 +/- 40.4</td>
<td>18 +/- 2.4</td>
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<tr>
<td>14</td>
<td>446 +/- 75.0</td>
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<tr>
<td>28</td>
<td>412 +/- 29.4</td>
<td>26 +/- 4.7</td>
</tr>
</tbody>
</table>

Table 2. Uptake rates of $^3$H-D-aspartate in untreated primary astrocytes. Uptake values in presence of 25 μM $^3$H-D-aspartate measured over 5 min at 30°C. Nonspecific uptake was corrected for by subtracting accumulated $^3$H-D-aspartate at 4°C. Shown are mean values that arise from duplicate readings of triplicate sample wells for independent experiments +/- SEM values for n = 5-11. p < 0.05 for day 1 verses day 14 groups.
Figure 9. Mean protein values for astrocyte cell cultures. Proteins were solubilized in 0.4M NaOH and values were obtained using the bicinchoninic acid method. Shown are mean values arising from triplicate readings per protein sample +/- SEM values for n = 5-11. No significant difference exists between groups.
Figure 10. Rates of TNB formation corresponding to standard amounts of GSH. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Each plot represents the mean rate of three identically treated wells. Plot represents a typical data set.
Table 3. Percent recovery of known amounts of GSH in 1% SSA on the microplate spectrophotometer. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Mean values arise from three identically treated wells and are expressed as percent recovery +/- SEM.

<table>
<thead>
<tr>
<th>GSH added (nmol)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>98.2 +/- 0.4</td>
</tr>
<tr>
<td>0.625</td>
<td>99.1 +/- 2.5</td>
</tr>
<tr>
<td>0.1563</td>
<td>97.7 +/- 2.2</td>
</tr>
<tr>
<td>0.039</td>
<td>99.1 +/- 2.7</td>
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</table>
Table 4. GSH values obtained from microplate and cuvette spectrophotometers in C6 glioma cells. The volume of the samples are 4-fold larger for the cuvette (200 µl) verses the microplate (50 µl) and total GSH values were adjusted accordingly. Data represents mean values that arise from triplicate readings from duplicate sample wells. 4-S-CPG (100 µM), BSO (500 µM). No significant difference exists between values for the two methods.
Figure 11. Levels of GSH in untreated primary astrocytes. On the day of the assay primary astrocytes are harvested at 22-50 days old (1-28 days post-plating), homogenized in 1% sulfosalicylic acid (SSA), microfuged for 1 min, and kept at 4°C until use. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Mean values arise from duplicate readings of three identically treated wells for each experiment and are expressed as nmol/mg +/- SEM for n = 3-5. No significant difference exists between groups.
Figure 12. The time course of BSO-mediated loss of GSH. Primary astrocytes were treated with 500µM BSO at 0 hrs and subsequently harvested at 0, 4, 8, 12, and 20 hrs, homogenized in 1% sulfosalicylic acid (SSA), microfuged for 1 min, and kept at 4°C until use. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Individual data points represent mean values that arise from duplicate readings from duplicate sample wells. Plot shown represents a typical data set.
Figure 13. Levels of GSH in BSO-treated astrocytes. On the day of the assay primary astrocytes are harvested at 22-50 days old (1-28 days post-plating), homogenized in 1% sulfosalicylic acid (SSA), microfuged for 1 min, and kept at 4°C until use. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Mean values arise from duplicate readings of three identically treated wells for each experiment and are expressed as nmol/mg +/- SEM for n = 3-5. No significant difference exists between groups.
<table>
<thead>
<tr>
<th>Time (experimental day)</th>
<th>GSH (nmol/mg) Control</th>
<th>GSH (nmol/mg) BSO</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.59 +/- 0.77</td>
<td>1.64 +/- 0.29</td>
<td>17 +/- 1.8</td>
</tr>
<tr>
<td>7</td>
<td>8.34 +/- 0.29</td>
<td>1.34 +/- 0.12</td>
<td>16 +/- 1.3</td>
</tr>
<tr>
<td>14</td>
<td>6.56 +/- 0.91</td>
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<td>13 +/- 2.2</td>
</tr>
<tr>
<td>28</td>
<td>8.24 +/- 2.04</td>
<td>0.99 +/- 0.42</td>
<td>11 +/- 3.2</td>
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</table>

Table 5. Summary of GSH levels in untreated and BSO-treated astrocytes. On day of assay primary astrocytes are harvested at 22-50 days old (1-28 days post-plating), homogenized in 1% sulfosalicylic acid (SSA), microfuged for 1 min, and kept at 4°C until use. Above data was obtained by monitoring the rate of TNB production at 405 nm for 5 minutes and represents total glutathione content. Mean values arise from duplicate readings of three identically treated wells for each experiment and are expressed as nmol/mg +/- SEM for n = 3-5. Day 1 (p < 0.0001), Day 7 (p = 0.0004), Day 14 (p = 0.005), Day 28 (p = 0.0003) for untreated verses BSO-treated groups.
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>BSO (µM)</th>
<th>Rate (pmol/min/mg)</th>
<th>% of control uptake</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>201 +/- 34.5</td>
<td>100</td>
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<td>500</td>
<td>204 +/- 38.4</td>
<td>94 +/- 2.8</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>359 +/- 39.1</td>
<td>100</td>
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<td>500</td>
<td>334 +/- 41.2</td>
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<td>14</td>
<td>0</td>
<td>419 +/- 75.3</td>
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<td></td>
<td>500</td>
<td>341 +/- 50.6</td>
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<tr>
<td></td>
<td>500</td>
<td>366 +/- 29.0</td>
<td>90 +/- 5.4</td>
</tr>
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</table>

Table 6. Uptake rates of $^3$H-D-aspartate in BSO (500µM) treated astrocytes. Uptake values in presence of 25 µM $^3$H-D-aspartate measured over 5 min at 30°C. BSO was administered with DMEM/F12 w/ 10% FBS feeding every fourth day in culture. Shown are mean values that arise from duplicate readings of triplicate sample wells for independent experiments +/- SEM values for n = 5-11. No statistically significant difference exists between rate or % control groups on each experimental day.
Table 7. Effects of acute challenge on uptake values of $^3$H-D-aspartate in untreated astrocytes. On day of assay astrocytes are removed from 37°C incubation, media is removed and replaced with assay buffer and insulted with X/XO (500μM/5mU/ml FV) or H$_2$O$_2$ (50μM FV), incubated at 37°C for 15 minutes. Uptake values in presence of 25 μM $^3$H-D-aspartate measured over 5 min at 30°C. Shown are mean percentages that arise from pooled percent control values calculated from duplicate readings of duplicate sample wells for independent experiments +/- SEM values for n = 5-7. No statistically significant difference exists between insult groups.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Insult</th>
<th>Rate (pmol/min/mg)</th>
<th>% of Control Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>212 +/- 33.0</td>
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<td>H$_2$O$_2$</td>
<td>189 +/- 35.7</td>
<td>81 +/- 5.34</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>253 +/- 39.1</td>
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<tr>
<td></td>
<td>X/XO</td>
<td>169 +/- 42.8</td>
<td>70 +/- 5.79</td>
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<td>Control</td>
<td>284 +/- 32.7</td>
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<tr>
<td></td>
<td>H$_2$O$_2$</td>
<td>201 +/- 23.4</td>
<td>71 +/- 6.35</td>
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<td>Control</td>
<td>206 +/- 31.4</td>
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<tr>
<td></td>
<td>X/XO</td>
<td>139 +/- 11.1</td>
<td>59 +/- 5.86</td>
</tr>
<tr>
<td>Time (days)</td>
<td>Insult</td>
<td>Rate (pmol/min/mg)</td>
<td>% of Control Uptake</td>
</tr>
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</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>230 +/- 43.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>H$_2$O$_2$</td>
<td>141 +/- 29.0</td>
<td>67 +/- 11.7</td>
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<td>Control</td>
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<tr>
<td></td>
<td>X/XO</td>
<td>111 +/- 8.4</td>
<td>67 +/- 3.06</td>
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Table 8. Effects of acute challenge on uptake values of $^3$H-D-aspartate in BSO (500μM) treated astrocytes. BSO was administered with DMEM/F12 w/ 10% FBS feeding every fourth day in culture. On day of assay astrocytes are removed from 37°C incubation, media is removed and replaced with assay buffer and insulted with X/XO (500μM/5mU/ml FV) or H$_2$O$_2$ (50μM FV), incubated at 37°C for 15 minutes. Uptake values in presence of 25 μM $^3$H-D-aspartate measured over 5 min at 30°C. Shown are mean percentages that arise from pooled percent control values calculated from duplicate readings of duplicate sample wells for independent experiments +/- SEM values for n = 5-7. No statistically significant difference exists between insult groups.
Figure 14. Summary of uptake values of $^3$H-D-aspartate in untreated and BSO (500μM) treated astrocytes following acute oxidant exposure. BSO was administered with DMEM/F12 w/ 10% FBS feeding every fourth day in culture. On day of assay astrocytes are removed from 37°C incubation, media is removed and replaced with assay buffer and insulted with X/XO (500μM/5mU/ml FV) or H$_2$O$_2$ (50μM FV), incubated at 37°C for 15 minutes. Uptake values in presence of 25 μM $^3$H-D-aspartate measured over 5 min at 30°C. Shown are mean percentages that arise from pooled percent control values calculated from duplicate readings of duplicate sample wells for independent experiments +/- SEM values for n = 5-7. No statistically significant difference exists between insult groups.
Figure 15. Tryptophan fluorescence in BSO (500µM) treated astrocytes. BSO was administered with DMEM/F12 w/ 10% FBS feeding every fourth day in culture. Following protein determination, equivalent amounts of protein were centrifuged on a high-speed microfuge, resuspended in 1% SDS, re-homogenized, and relative fluorescence determined at ex 280Å em 345Å. Shown are mean values of percent of controls for multiple readings from individual experiments expressed as % RFU +/- SEM for n = 3-5. No statistically significant difference exists between groups.
Figure 16. Tryptophan fluorescence in acutely challenged untreated astrocytes. Astrocytes are exposed to either X/XO (500μM/5mU/ml FV) or H₂O₂ (50μM FV) at 22-29 days old (day 1 and 7 post-plating) and allowed to incubate at 37°C for 15 minutes as in uptake experiments. Following protein determination, equivalent amounts of protein were centrifuged on a high-speed microfuge, resuspended in 1% SDS, re-homogenized, and relative fluorescence determined at ex 280λ and max em (405-410λ). Shown are mean values of percent of controls for multiple readings from individual experiments expressed in RFU +/- SEM for n = 4. No significant difference exists between groups.
Figure 17. Tryptophan fluorescence in acutely challenged and BSO (500μM) treated astrocytes. Astrocytes are exposed to either X/XO (500μM/5mU/ml FV) or H₂O₂ (50μM FV) at 22-29 days old (day 1 and 7 post-plating) and allowed to incubate at 37°C for 15 minutes as in uptake experiments. Following protein determination, equivalent amounts of protein were centrifuged on a high-speed microfuge, resuspended in 1% SDS, re-homogenized, and relative fluorescence determined at ex 280μ and max em (405-410μ). Shown are mean values of percent of controls for multiple readings from individual experiments expressed in RFU +/- SEM for n = 4. No statistically significant difference exists between groups.
Figure 18. Tryptophan fluorescence summary in untreated and BSO (500μM) treated astrocytes exposed to an acute oxidant challenge. Astrocytes (BSO treated or un-treated) are exposed to either X/XO (500μM/5mU/ml FV) or H₂O₂ (50μM FV) at 22-29 days old (day 1 and 7 post-plating) and allowed to incubate at 37°C for 15 minutes as in uptake experiments. Shown are mean values of percent of controls for multiple readings from individual experiments expressed as % RFU +/- SEM for n = 6. No statistically significant difference exists between groups.
Discussion

The uptake of glutamate through the glutamate transporters is the only known mechanism (aside from diffusion) for clearing glutamate from the synaptic space. The present study was undertaken to examine the sensitivity of glutamate transport to mild oxidative stress induced as a consequence of glutathione depletion. Central to this project are two fundamental observations. The first is that glutamate transporters provide a link between excitotoxicity and ROS production. Excessive oxidative stress has been shown to inhibit glutamate transport by direct action on the transporters (Piani et al., 1993; Volterra et al., 1994; Trotti et al., 1997; Rao et al., 2003). Following transport inhibition, extracellular glutamate levels increase and can accumulate to excitotoxic levels. Excitotoxic levels of glutamate, through a calcium dependent manner, can lead to ROS production (Dugan et al., 1995; Reynolds et al., 1995; Lancelot et al., 1998; Rao et al., 2003). A common approach to study the relationship between excitotoxicity and oxidative damage has been to introduce oxidants at levels high enough to induce excitotoxicity and death in glia and neurons.

In contrast, we chose to examine the effects of endogenously produced oxidative stress on glutamate transport. Because the majority of glutamate transport is thought to be mediated by glial cells, astrocytes were chosen as our experimental system. Under the conditions employed, primary astrocytes expressed the GLAST subtype of glutamate transporters. All of the glutamate transporters examined (GLAST, GLT-1, EAAC1) have been reported to be equally susceptible to ROS-mediated inactivation as a result of oxidation of conserved cysteine residues (Trotti et al., 1997; Trotti et al., 1998). The
conversion of cysteine residues from the reduced to the oxidized state is thought to influence the Vmax of transport, but not the apparent affinity for substrate (Km).

The second observation is that glutathione serves as a significant cellular antioxidant and reducing agent (Orlowski et al., 1976; Raps et al., 1989; Meister, 1995; Ben-Yoseph et al., 1996). In studies by Volterra et al. the application of 500μM/50mU/ml X/XO and 500μM H2O2 to astrocytes resulted in the inhibition of glutamate uptake that was prevented and partially restored with GSH and the disulphide reducing agent DTT. Interestingly, the transport activity could not be prevented or restored with lipophilic antioxidants (vitamin E, lipophilic iron chelators, MP) nor were any lipid peroxidation products present (Volterra et al., 1994). For this reason, we hypothesized that the glutamate transporter proteins may rely on GSH for protection from oxidative damage.

In the CNS, glial cells are thought to be a major source of GSH synthesis. The primary astrocytes used for this study contained stable levels of GSH throughout the experimental time course. To assess the sensitivity of the glutamate transporters to endogenous levels of ROS produced through normal cellular metabolism, intracellular GSH levels were depleted with BSO and the uptake of D-aspartate was measured. Initial studies confirmed both the effectiveness of BSO as an inhibitor of GSH synthesis and the lack of a substantial experimental introduction of exogenous GSH in the astrocyte cultures (see Results). The potential sensitivity of transport activity was then further examined by exposing cells to non-lethal levels of well known exogenous oxidants. The ability to attenuate transport through the EAATs through relatively high levels of ROS exposure has been demonstrated (Piani et al., 1993; Volterra et al., 1994; Trotti et al., 1997; Rao et al., 2003). It was hypothesized that if the EAATs were insensitive to
oxidative inactivation by GSH depletion alone, that the transporters may exhibit an increased vulnerability to exogenous oxidants. While the LD50 for H$_2$O$_2$ depends on many factors including cell type, length of exposure, transition metals present, media constituents, the amount of H$_2$O$_2$ employed in this study was substantially lower than that shown to induce cell death and is referred to as sub-lethal or moderate. Initial values were chosen that previously demonstrated measurable effects on uptake (~80% of control) in similar cultures in our lab (Webster, 1998). Experiments with astrocytes indicate that oxidant levels as high as 500μM H$_2$O$_2$ and 500μM/50mU/ml X/XO are capable of inhibiting glutamate uptake without demonstrating a significant release of LDH (Volterra et al., 1994). The assumption was made, based on this information, that moderate insult would be achieved with 50μM H$_2$O$_2$ and 500μM/5mU/ml X/XO. Interestingly, as far as H$_2$O$_2$ is concerned, the value used in our experiments approximates that which could be measured in freshly voided human urine (Halliwell, 2000). In the present experiments, cells were washed prior to adding oxidants so that any GSH in the cell media of untreated cells would be removed in this step. Therefore, upon addition of the assay buffer and oxidant, the extracellular conditions of the untreated astrocytes and those treated with BSO were identical.

Oxidative insults far exceeding those used in this study have previously been shown to inhibit uptake, and is primarily thought to be due to protein damage (Volterra et al., 1994; Drukarch et al., 1997; Trotti et al., 1997). The fluorescence of tryptophan residues are commonly used to monitor changes in protein structure (Jang et al., 1989; Kleinschmidt et al., 1999; Ladokhin et al., 2000; Hansen et al., 2002; Mozo-Villarias, 2002). The changes in proteins following exposure to oxidants are significant enough to
elicit declines in tryptophan fluorescence (Davies et al., 1987; Jones et al., 1987; LeBel et al., 1991). It has been shown that the pyrrol ring of tryptophan is the most susceptible to oxidative attack resulting in the formation of a number of degradation products (Simat et al., 1998). While tryptophan degradation and the concurrent change in fluorescence is not an exclusive phenomena in terms of protein oxidation, it does serve as an established indicator for insult studies (Davies et al., 1987).

We found that decreasing intracellular GSH levels does not affect the uptake rates of D-aspartate through GLAST. This suggests that ROS produced endogenously during normal respiration in the primary astrocytes do not accumulate to a level that are deleterious to the transport process under conditions of GSH depletion. This could mean that under the conditions used, the metabolic rate of astrocytes may not have been sufficient to generate a level of ROS capable of altering the transport process. It is possible that certain energy dependent processes in astrocytes (glutamate uptake, glutamine synthesis, and glutathione synthesis) may be limited in the present study due to both the low level of glutamate in the media (50 μM) and the presence of BSO. Therefore, as a consequence of a lowered metabolic rate, endogenous ROS generation may be diminished.

Alternatively, it is also possible that the cells are generating toxic levels of ROS endogenously but are protected through mechanisms other than GSH. It has been shown that removing the scavenging capacity of GSH does not always lead to pathology or cell death. For example, acatalasaemia, the deletion of GSH-peroxidase, appears to produce no specific phenotype (Halliwell, 2000). Therefore, it would not be surprising if astrocytes were capable of compensating for lowered GSH levels and oxidative stress. In
fact, when astrocytes are treated with 500μM BSO, GSH levels are lowered (10% of control) but no accumulation of NO* or H$_2$O$_2$ is observed (McNaught et al., 2000). Not only do astrocytes appear to be under a low amount of stress during GSH depletion, but when treated with various amounts of H$_2$O$_2$ under normal conditions they are shown to exhibit as much as a 6-fold higher resistance to cell death than neurons (Ben-Yoseph et al., 1996). These observations may be explained in part by the up-regulation of the pentose phosphate pathway in astrocytes verses neurons (Ben-Yoseph et al., 1996) as well as antioxidant enzyme upregulation (MnSOD, GPx, catalase) that in some cases is known to occur in cells exposed to various levels of oxidative stress (Rohrdanz, 2001).

Another possibility is that the activity of the glutamate transporters is not sensitive to oxidative insult as initially thought. This does not appear to be the case, however, as transport activity was inhibited by moderate levels of exogenously applied oxidants (50μM H$_2$O$_2$ and 500μM/5mU/ml X/XO). When astrocytes were depleted of GSH and treated with the same oxidative challenge, transport was not further attenuated. This means that even when oxidant levels capable of altering transport are applied to astrocytes, depleting intracellular GSH levels does not increase the vulnerability of the glutamate transporters.

Taken together these results suggest that under the conditions stated, the availability of GSH is not likely important for the protection of the transporters from endogenously produced or exogenously administered oxidants. Similar results have been observed by others (Piani et al., 1993). One explanation is that the ability of GSH to scavenge free radicals and reduce thiol groups may be limited depending on the predominant site of oxidative damage (intracellular or extracellular). The effect of the
oxidants then must be considered in terms of oxidatively vulnerable sites on the transporter proteins. To address the susceptibility of GLAST, we must consider both the direct and indirect modifications that could result from ROS treatment. A change in protein structure and possibly function can result from the direct oxidation of proteins from ROS and lipid-propagated peroxidation. Additionally, changes in membrane integrity and essential cellular proteins can indirectly modify the function of the glutamate transporters.

The most direct form of damage would be to the transport protein itself, through the modification of primary structure accomplished via a variety of amino acid alterations. The highly specific and selective nature of proteins can be compromised greatly by seemingly minor changes in amino acid moieties. Changes in protein conformation, folding, and subsequently, function, can result from ROS damage to amino acids. One of the most common examples of this is the oxidation of sulfhydryl groups on cysteine residues resulting in disulphide bond formation. Three of the glutamate transporter subtypes (GLAST, GLT-1, EAAC1) have been shown to be equally inhibited by the direct action of oxidants and despite the number of cysteine moieties contained in each subtype (three in GLAST (Storck et al., 1992), nine in GLT1(Pines et al., 1992), and six in EAAC1(Kanai et al., 1992)) it is believed that they share a common regulatory mechanism governed by the chemical redox state of cysteine groups (Trotti et al., 1997; Trotti et al., 1998 for review). And because two conserved cysteine groups exist between all glutamate transporters, this very well could be the most significant susceptible site for direct effects on function.
In GLAST, C186 and C375 are the conserved cysteine residues (accession numbers: GLAST, P24942; GLT-1, P31596; EAAC1, P31597). According to the current consensus on glutamate transporter topology, at least three extracellular loops exist and may contain redox sensitive target sites (for review see Danbolt, 2001). Interestingly, C186 (Wahle et al., 1996) and C375 (Seal et al., 2000) in GLAST are both thought to be located extracellularly. This information may help to explain why GLAST transport activity in untreated cells (containing 6-fold more intracellular GSH content than BSO-treated cells) and those treated with BSO was equally affected by the introduction of oxidants. It is possible that the hydrogen peroxide and products xanthine/xanthine oxidase reaction had access to extracellular cysteine groups and intracellular GSH is not exported from the untreated cells in sufficient amounts to protect this portion of the transporter. Even though the membrane permeability of the oxidants would grant them access to the intracellular space, as long as the sites of vulnerability of GLAST are extracellular, it is possible that intracellular protectant mechanisms are not relevant. Additionally, unlike the cultures chronically depleted of GSH, it is not likely that astrocytes exposed to oxidants for 15-minutes are capable of opposing the insult through upregulation of various antioxidant enzymes (Piani et al., 1993).

Extracellular oxidation of cysteine groups on GLAST is one but not the only mechanism whereby transport may be compromised following exposure to oxidants. Direct modifications to the membrane or transporter-dependent proteins may indirectly affect the activity of the transporters. Membrane damage from lipid peroxidation involves hydroxy radical initiation of the self-propagating chain whereby the abstraction of a hydrogen atom from a polyunsaturated fatty acid side chain forms carbon centered...
lipid radicals. Carbon centered radicals most likely react with \( \text{O}_2 \) in vivo to form peroxyl radicals which interact with nearby lipids to form more lipid radicals, peroxyl radicals, lipid hydroperoxides, and reactive aldehydes, propagating the oxidative chain (Halliwell, 1992). Additionally, in the presence of transition metal ions, hydroperoxides can generate lipid peroxyls and the chain can continue. Membrane proteins can be damaged directly by the products of the lipid peroxidation chain (peroxyl radicals and aldehydes) or indirectly through changes in membrane fluidity, altered permeability of the membrane to ions, and decreased ATPase function as a result of increased oxidation of membrane lipids (Halliwell, 1992; Juurlink et al., 1998). Undoubtedly, a change in \( \text{Na}^+ \), \( \text{K}^+ \), and \( \text{H}^+ \) permeability can disrupt the electrochemical gradients that are responsible for the transport of glutamate, and lowered ATP levels can precipitate gradient failure. Maintaining a glutamate gradient of several thousand-fold is highly energy dependent as can be seen in episodes of ischemia where oxygen and ATP levels are diminished and neuron cell death occurs. An energy coupled ionic gradient is not the only requirement for transport, however, thermodynamically uncoupled ion gradients, pH changes (lower inside and higher outside), as well as temperature all affect the transport process (Danbolt, 2001 for review).

Membrane disruption through lipid peroxidation is clearly a deleterious process to glutamate transport. However, lipid peroxidation is unlikely to occur independently of protein damage, but protein damage has been shown to occur independently of lipid damage (Volterra et al., 1994). Therefore, in addition to direct protein damage of the transporters, there could exist situations where direct damage to adjacent or functionally dependent proteins indirectly alters glutamate transport activity. For instance, there is
accumulating evidence that the glutamate transporters, similar to other membrane
transporter and receptors, may be kept in place by anchoring proteins. These proteins are
shown to bind to the C-terminal ends of the transporters (intracellularly) and serve to
maintain the specialized localization of the transporters and possibly play a role in
transporter trafficking. The intracellular C-terminus of EAAT5 has a protein binding
motif similar to a binding domain on NMDA receptors, GLAST has a C-terminal binding
site that modulates its affinity for glutamate, and the C-terminal of GLT-1 is an oxidation
site mediated by mutant superoxide dismutase (Danbolt, 2001). Therefore, transporter
expression and activity could be compromised through the alteration of associated
proteins.

Speculation upon the source of oxidative stress is limited to the technique used to qualify
the resultant damage. Because ROS are normally reactive and labile, it is often difficult to
measure them directly. The direct oxidation of proteins, however, can modify the
ultraviolet spectrum and fluorescence of protein, or more often, render it more reactive
with certain compounds that can subsequently be assayed. In this way, more stabile
oxidatively modified products of proteins and lipids are measured as markers of oxidative
damage. Protein carbonylation (Levine, 1990; Chevion, 2000), oxidation products of
tryptophan, histidine, lysine, methionine, and phenylalanine (Simat et al., 1998; Chevion,
2000), lipid peroxides (Gotz, 1993; Shwaery, 1999), 4-hydroxynonenol (Rohn et al.,
1998), and oxidant-sensing fluorescent probes (Hempel, 1999) serve as reliable markers
of oxidative stress. In the results presented above, tryptophan fluorescence did not change
relative to the decline in intracellular GSH over the 28-day course, as well as to mild acute challenge in both untreated and BSO-treated astrocytes.

The results of the tryptophan fluorescence experiments indicate that tryptophan oxidation did not occur to a significant degree in astrocyte proteins exposed to a mild oxidative insult, proving the null hypothesis. This assumes that the amounts of H$_2$O$_2$ and X/XO applied to astrocytes were unable to oxidize tryptophan moieties that face extracellularly, did not gain access to the intracellular environment, or if they did, were insufficient for measurable oxidative damage. Concentration is not the only condition that may explain the outcome however. The additions of H$_2$O$_2$ were allowed to incubate at 37°C for 15 minutes, this may not have been long enough to see changes in the cellular proteins. Simat et al has shown that temperature and an extended time course of up to 6 hours are determinants for measuring a loss tryptophan fluorescence in peptides and proteins exposed to 0.2M H$_2$O$_2$ (Simat et al., 1998).

The contribution of antioxidants other than GSH toward ROS scavenging may have limited the oxidative stress that cells were under and consequently diminished any measurable protein fluorescence. Cu,Zn-SOD and Mn-SOD may serve as additional enzymatic systems for combating extra free radicals produced during respiration in the absence of GSH. The product of SOD, H$_2$O$_2$ remains a possible mediator of protein damage and absence of GSH prevents glutathione peroxidase from facilitating its removal. However, other peroxidases in the brain exist, and thioredoxin-dependent peroxidases have been linked to preventing ischemic oxidative damage and cell death (Halliwell, 2001). Intracellularly, glia and neurons concentrate ascorbate to millimolar levels and this too may render mild oxidative insult (assuming it gets in) ineffective.
Lipid protection is often thought to be mediated through α-tocopherol, and while important for quenching lipid peroxidation chain events, its role is not fully understood as normal levels in the CNS are not linked with a preventative effect on aging, Alzheimer's, or Parkinson's disease (Halliwell, 2001). In this study, the normal production of GSH through functional transport of glutamate and cystine serves as the major peroxide scavenging reductant in the samples not treated with BSO.

This does not imply however, that in the absence of observed changes in tryptophan fluorescence, the incubation time or concentration was insufficient, or that the cellular environment was sufficiently protective. It is likely that some constituents within the insult regimen serve to limit the oxidation of tryptophan. In experiments where cells were treated with the free radical generating system X/XO, uric acid with the concurrent generation of one H₂O₂ molecule and one superoxide ion are produced. Uric acid as well as the main constituent of the assay buffer, HEPES, are both known to protect against tryptophan loss in studies with free radical insult to BSA (Davies et al., 1987). Just because the insult applied may be insufficient to cause oxidation of tryptophan residues or is undetectable for some other reason, it does not mean that inhibition of D-aspartate uptake in cells exposed to ROS is not the result of oxidative damage. As mentioned earlier, there are other markers of oxidative damage other than tryptophan fluorescence and these may better reveal the modifications that may have occurred to GLAST or other cellular proteins. In other words, protein damage likely occurred from H₂O₂ and X/XO treatment, however, the nature of the protein damage may be more sensitive or more specific than the technique used to resolve it. In this study, it appears D-aspartate uptake served as a more sensitive marker of oxidative stress than did tryptophan fluorescence.
The effects (direct and indirect) of the oxidants responsible for the decline in GLAST activity, appear to be independent of intracellular GSH levels. We conclude that the availability of GSH is probably not important for the protection of GLAST from endogenously produced or limited levels of exogenously administered oxidants because of the rate of ROS normally generated in cultured astrocytes, alternative compensatory mechanisms, or the inability of intracellular GSH to sufficiently protect the extracellular portion of the transporter from oxidative stress. It is likely that the protecting ability of GSH is dependent on where (intracellular or extracellular) the oxidants predominantly exert their effects. Sulfhydryl groups on the extracellular surface of GLAST may be inaccessible to intracellular GSH and thus be the most susceptible sites for oxidation following H$_2$O$_2$ and X/XO exposure. Because the decline in D-aspartate transport activity associated with untreated and BSO-treated astrocytes was not paralleled by tryptophan fluorescence, it appears that in this case transport activity served as a more reliable marker of oxidative stress than the more general marker.

The production of ROS and oxidative inactivation of the glutamate transporters have been associated with a number of neuropathologies. Consequently, a major strategy for the treatment of acute pathology and neurodegeneration has involved using antioxidant therapies to both increase protective enzymes and suppress oxidative stress. One interpretation of the current results may be that the antioxidant GSH is not a relevant therapeutic target for the protection of the transporters and their downstream links to excitotoxicity.
However, the often multifactorial nature of disease progression limits the validity of this conclusion. For any one disease state there may exist a number of possible risk factors that can independently lead to an identical pathology. Genetic polymorphisms in repair mechanisms, mitochondrial enzymes, and DNA repair mechanisms may have similar clinical manifestations but require very different therapies. For example, patients afflicted with ALS have responded to some antioxidant therapies and not others, while current research suggests that oxidative stress may not play a significant role in the etiology of the disease (Halliwell, 2001; Tortarolo et al., 2004). Additionally, the inability to separate cause from consequence in pathology further confounds the treatment process. One consequence in those afflicted with Parkinson's disease is lowered GSH levels. However, decreasing GSH levels does not reproduce Parkinson's disease (Danbolt, 2001; Halliwell, 2001). It is likely that the results of a focused study are better applied to a growing body of knowledge relating to a particular disease state than directly to a treatment paradigm.

The relevance of GSH in therapy remains unknown. Clearly, protecting the transporters from oxidative inactivation may limit the excitotoxicity cascade (Figure 5). Therefore, continued investigation of transport sensitivity to oxidative stress is required. A number of factors could influence the response of transport activity to oxidative stress, including the amount of ROS used, the time course of exposure (acute verses chronic), and the site of exposure. While previous studies have focused on acute exposure to high levels of ROS at a number of sites in the CNS, we chose to study chronic exposure to low levels of ROS, in one cell type. In order to better understand the dependence of glial transport on GSH, the role of neurons must be considered. In a co-culture system
consisting of astrocytes and neurons, the metabolic rate of astrocytes may increase as a result of enhanced growth and activity, leading to higher levels of ROS, and consequently increase the reliance of astrocytes on the antioxidant qualities of GSH. This effect would be compounded by the high energy demands of neurons (transport, synthesis, storage, release) which may lead to increased endogenous oxidative stress and result in even higher rates of GSH turnover in the co-culture system. To ascertain the role of GSH in this co-culture scenario it would be advantageous to not only observe the effects of depletion but also to use GSH to prevent or recover any effects on transport that may occur. Depending on the reducing reagents used, this may also address the location of the damage that may occur from endogenous oxidative stress to the transporters. As mentioned above, there also is the possibility that mechanisms other than GSH may serve to compensate for oxidative stress in astrocytes. In vivo and in vitro observations indicate that neurons are more susceptible to excitotoxicity and ROS than astrocytes and undergo cell death earlier and in higher numbers (Ciani et al., 1996; Magistretti, 2003; Rao et al., 2003). Neuronal morbidity could be used to help assign priorities to various protectant mechanisms under investigation.

Glutamate transport is thought to regulate the interaction of two destructive processes: excitotoxicity and ROS formation. Further insight into the sensitivity of the transporters to ROS-mediated damage is necessary for understanding pathogenic events resulting from aberrant glutamate neurotransmission.
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