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Cellular interplay between neurons and glia: toward a comprehensive mechanism for excitotoxic neuronal loss in neurodegeneration

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Abstract

Astrocytes perform vital maintenance, functional enhancement, and protective roles for their associated neurons; however these same mechanisms may become deleterious for neurons under some conditions. In this review, we highlight two normally protective pathways, the endoplasmic reticulum (ER) stress response and an endogenous antioxidant response, which may become neurotoxic when activated in astrocytes during the inflammation associated with neurodegeneration. Stimulation of these multifaceted pathways affects a panoply of cellular processes. Of particular importance is the effect these pathways have on the homeostasis of the excitatory amino acid neurotransmitter, glutamate. The endogenous antioxidant response increases extracellular glutamate in the pursuit of making the cellular antioxidant, glutathione, by increasing expression of the xCT subunit of the cystine/glutamate antiporter. Meanwhile, inflammatory mediators such as TNFα reduce levels of membrane-bound glutamate scavenging proteins such as the excitatory amino acid transporters. Together, these cellular activities may result in a net increase in extracellular glutamate that could alter neuronal function and lead to excitotoxicity. Here we discuss the role of N-methyl-D-aspartate receptors, which, when excessively stimulated by glutamate, can cause neuronal dysfunction and loss via activation of calpains. While there are other pathways acting in concert or parallel to those we describe here, this review explores a rationale to explain how two protective mechanisms may result in neuronal loss during neurodegeneration.

Keywords

glutamate; endoplasmic reticulum stress; oxidative stress; brain; antioxidant response; Nrf2; PERK; calpain; NMDA receptor; integrated stress response; xCT; excitatory amino acid transporters

Introduction: Insults and Injury in Neurodegeneration

Slow, chronic, progressive damage and loss of neurons underlie the symptoms observed in neurodegenerative diseases including Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and HIV-associated dementia (HAD). Although the precise mechanisms of neuronal loss in these diseases have yet to be elucidated, evidence from human autopsy tissue, animal studies, and *in vitro* work demonstrate a prominent role for

*To Whom Correspondence Should Be Addressed: Kelly L. Jordan-Sciutto, PhD, 240 S. 40th St, Room 312 Levy Building, Department of Pathology, University of Pennsylvania, Philadelphia, PA 19104, phone: 215-898-4196, fax: 215-573-2050, e-mail: Jordan@path.dental.upenn.edu.

oxidative stress (Lipton et al., 1994; Ehret et al., 1996; Koutsilieri et al., 1997; Kruman et al., 1998; Malorni et al., 1998; Romero-Alvira and Roche, 1998; Mollace et al., 2001; Viviani et al., 2001). Endogenous agents that reduce cellular oxidative damage such as glutathione and catalase are depleted in patients with AD, PD, ALS and HAD (Sofic et al., 1992; Castagna et al., 1995; Sohal and Weindruch, 1996; Lovell et al., 1998; Choi et al., 2000; Sayre et al., 2001). By providing exogenous antioxidants or enzymes that destroy free radicals in animal models or *in vitro* models of AD, PD, and HAD (Kruman et al., 1998; Malorni et al., 1998; Floyd et al., 1999; Ramassamy et al., 1999; Wang and Xu, 2005) or as therapeutics in human trials (Anonymous, 1998; Muller et al., 2000; Shor-Posner et al., 2002; Turchan et al., 2003; Weinreb et al., 2004), neuronal loss can be attenuated. Though the sufficiency of these treatments in preventing neurodegeneration still remains unclear, these studies suggest that reduction in oxidative stress will mitigate disease progression.

Neuroinflammation can trigger oxidative stress and act as a prominent insult in AD, PD, ALS, and HAD. While there is evidence of microgliosis and astrogliosis in each of these neurodegenerative conditions, neuroinflammation is most closely associated with disease etiology and progression in HAD. Pathologic studies of the brains of patients with HAD suggest an inflammatory mechanism in the progression of this disease (Kaul et al., 2001; Garden et al., 2002; Ghorpade et al., 2003). Neuronal dysfunction and death in HAD is mediated by the release of neurotoxic factors from activated macrophages and microglia including, but not limited to, reactive oxygen species and excitatory amino acids (Pulliam et al., 1991; Yoshioka et al., 1995; Conant et al., 1998; Bagetta et al., 1999; Nath et al., 1999; Kaul et al., 2005). Moreover, these factors stimulate astrocytes and neurons to release additional pro-inflammatory factors, perpetuating the inflammatory state in the CNS, and potentially exacerbating neuronal damage (Gonzalez-Scarano and Martin-Garcia, 2005). Soluble macrophage factors associated with neurologic damage in the CNS of AIDS patients include excitotoxins (i.e. quinolinic acid, glutamate), reactive oxygen species (ROS), cytokines, chemokines and neurotrophic factors (Masliah et al., 1992; Brouwers et al., 1993; Gelbard et al., 1994; Pulliam et al., 1994; Crowe, 1995; Achim and Wiley, 1996; Giulian et al., 1996; Pulliam et al., 1996; Heyes et al., 1998; Sanders et al., 1998). How neurons “interpret” the conflicting signaling environment in HAD or other neurodegenerative diseases ultimately determines viability. Factors such as neurotrophic factor and chemokines have been reported to activate survival pathways in neurons as part of their signaling cascades, while ROS and cytokines can induce death via apoptosis or necrosis (Nicotera et al., 1997; D’Mello, 1998; Pulliam et al., 1998; Bibel and Barde, 2000; Boldyrev, 2000; Holmin and Mathiesen, 2000; Takikita et al., 2001; Annunziato et al., 2003; Barzilai et al., 2003). Understanding the impact of these factors with disparate effects on neuronal viability is essential to unraveling the mechanisms that lead to neurodegeneration.

As important as investigating the mechanisms of neuronal loss in neurodegeneration is the role of neuronal support cells in neuroprotection and function, with particular emphasis on the astrocytes. Astrocytes not only support neuronal survival, but provide significant support of synaptic function by modulating neurotransmitter reuptake, neurotransmitter metabolism, and neurotrophic factor release (reviewed in (Benarroch, 2005)). Astrocytes have been shown to be sufficient to provide neuronal protection from oxidative stress, an insult associated with neurodegeneration (Shih et al., 2003; Shih et al., 2005). The cellular pathway by which astrocytes protect from oxidative stress is part of the integrated stress response (Cullinan et al., 2003; Shih et al., 2003; Shih et al., 2005; Cullinan and Diehl, 2006), a recently described mechanisms made up of at least two pathways important for maintenance of cellular homeostasis. In this review, we will highlight the importance of these protective pathways in the interplay between neurons and astrocytes under neuroinflammatory conditions and propose a model by which protective pathways can be subverted to neurotoxic pathways culminating in neuronal dysfunction and death.

Altered Astrocyte Functions in Response to Neurodegenerative Insults

Integrated Stress Response (ISR)

In the past several years, it has become clear that there is a common mechanism by which cells respond to various stresses. This mechanism has been coined the integrated stress response (ISR), also referred to as the endoplasmic reticulum (ER) stress response and the unfolded protein response (UPR). Although this response has been linked to misfolded or misprocessed proteins (Shen et al., 2004), it is well characterized as part of the cellular response to multiple stresses including oxidative stress, altered lipid metabolism, viral infection, hypoxia, and nutrient deprivation, several of which are triggered in AD, PD, ALS and HAD (Yu et al., 1999; Barbosa-Tessmann et al., 2000; Kudo et al., 2002; Ryu et al., 2002; Ledoux et al., 2003; Katayama et al., 2004; Isler et al., 2005; Mao et al., 2005; Xue et al., 2005; Blais et al., 2006; Fels and Koumenis, 2006; Kikuchi et al., 2006; Wei et al., 2006).

This coordinated response to stress is currently characterized by three pathways, each of which is regulated by its own initiator that is activated upon insult exposure. Specifically, the three ISR initiators are pancreatic ER kinase (PERK), inositol require enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) (Figure 1). In the endoplasmic reticulum, PERK, IRE1α, and ATF6 are kept in inactive forms through complex formation with the chaperone protein BiP (binding protein, also known as GRP78) (Bertolotti et al., 2000;Okamura et al., 2000;Shen et al., 2004). Induction of stress disrupts interaction between BiP and the three ISR regulators (Hurtley and Helenius, 1989) permitting activation of these key regulators. When free of BiP, ATF6 migrates to the transgolgi where the 50 kD N-terminal cytosolic fragment is released by proteolysis, which then translocates to the nucleus and activates expression of genes containing the ER stress response element (ERSE) in their promoter regions (Haze et al., 1999;Li et al., 2000;Ye et al., 2000;Yoshida et al., 2001;Lee et al., 2002;Shen et al., 2004). Disruption of BiP:IRE1α interaction permits dimerization of IRE1α, a serine/threonine kinase with riboendonuclease activity (Nikawa and Yamashita, 1992;Cox et al., 1993). When dimerized, IRE1α autophosphorylates, activating a splicing event that removes an inactivating intron from the unspliced XBP1 (uXBP1) mRNA (Kawahara et al., 1997;Sidrauski and Walter, 1997). Removal of this intron permits efficient translation of the spliced XBP1 (sXBP1) protein, which also activates the ERSE. Finally, disruption of BiP and PERK interactions allows multimerization of PERK, triggering its kinase activity, thereby resulting in autophosphorylation and phosphorylation of eukaryotic initiation factor 2 α (eIF2α) (Shi et al., 1998;Harding et al., 1999;Harding et al., 2000).

Of specific relevance for the PERK pathway, phosphorylation of eIF2α leads to inhibition of translation initiation for a majority of cellular proteins (Shi et al., 1998; Harding et al., 1999; Harding et al., 2000) while enhancing translation of select proteins whose functions are to facilitate reestablishment of cellular homeostasis (Fawcett et al., 1999; Harding et al., 2000). eIF2α is a GTP binding protein that delivers the initiator methionine-tRNA to the 40S ribosome (Reviewed in (Brostrom and Brostrom, 1998)). During translation initiation, the eIF2α-bound GTP is hydrolyzed to GDP. Phosphorylation of eIF2α stabilizes the interaction between eIF2α/GDP/eIF2 preventing exchange of the GDP for GTP. Thus, when eIF2α is phosphorylated, translation re-initiation is favored over new initiation, increasing translation of mRNAs with multiple micro open reading frames (called μORF) in their 5′ coding regions. Among the mRNAs translated is the activating transcription factor 4 (ATF4) transcript whose gene product also transactivates expression of the ERSE (Fawcett et al., 1999; Harding et al., 2000). The ERSE is found in the promoter of chaperone genes including BiP and an apoptotic regulator, CHOP (CCAAT enhancer binding protein homologous protein; reviewed in (Kaufman, 1999)). Activation of ERSE genes, including BiP and CHOP, are blunted in $PERK^{-/-}$ cells suggesting that PERK activity contributes significantly to ISR activation (Harding et al., 2000; Novoa et al., 2001).

In addition to inhibition of translation and activation of the ERSE, PERK can modify gene expression through phosphorylation of the Nrf2 transcription factor (Nuclear factor E2-related factor 2), a key regulator of redox homeostasis (Cullinan et al., 2003). In astrocytes, Nrf2 has been shown to upregulate expression of antioxidant genes in response to toxins and is required for protection against oxidative stress (Johnson et al., 2002; Lee et al., 2003; Kraft et al., 2004; Qiang et al., 2004). Nrf2 is normally sequestered in the cytoplasm, but in response to oxidative stress and/or phosphorylation by PERK, Nrf2 translocates to the nucleus and activates expression of detoxifying enzymes and antioxidants (Itoh et al., 1999). Thus, PERK contributes to the activation of the antioxidant pathway (Cullinan and Diehl, 2004).

Antioxidant Pathway of the ISR: Nrf2

When triggered, the antioxidant response can neutralize free radicals, preventing the deleterious effects these radicals have on cells such as direct oxidation of bases within DNA, reaction with unsaturated lipids leading to cellular membrane breakdown, or oxidative modification of amino acids. The enzymes responsible for the endogenous antioxidant response are regulated predominantly by transcriptional activation via Nrf2 (Itoh et al., 1997; Kwak et al., 2001; McMahon et al., 2001). The Nrf2 transcription factor is normally bound in the cytoplasm by the Kelch-like ECH-associated protein 1(Keap1) (Itoh et al., 1999). In response to oxidative stress, sulfhydryl groups on Keap1 become oxidized releasing Nrf2 permitting its translocation into the nucleus (Dinkova-Kostova et al., 2002). Release of Nrf2 from Keap1 is regulated, at least in part, by protein kinase C phosphorylation (Huang et al., 2000, 2002; Bloom and Jaiswal, 2003; Numazawa et al., 2003). These findings implicate Keap1 as a "sensor" of cellular stress that can be activated by oxidative stress and/or activation of second messenger cascades.

Upon translocation to the nucleus, Nrf2 increases transcription of genes containing the cis-acting regulatory antioxidant response element (ARE; also referred to as the electrophilic response element, EpRE) in the promoter region with core sequence 5′-GTGACnnnGC-3′ (Johnson et al., 2002; van Muiswinkel and Kuiperij, 2005). Such ARE-containing genes include antioxidant response enzymes and phase II detoxifying genes which play an essential role in protecting cells against oxidative stress by facilitating the inactivation and elimination of the original insult (Johnson et al., 2002). Among the gene products regulated at the transcriptional level by Nrf2 are the enzymes for glutathione synthesis, an essential antioxidant in the brain. Among the enzymes contributing to glutathione synthesis is the xCT subunit of the glutamate/cystine antiporter, which also contributes to glutamate homeostasis.

Studies have suggested Nrf2 regulation has importance in various neurodegenerative conditions. In pharmacologically induced Parkinsonian mice, induction of Nrf2 can alleviate symptoms (Burton et al., 2006). These findings correspond with work that has demonstrated that downregulation of DJ-1, a PD-associated protein, leads to decreased Nrf2 by affecting its stabilization with Keap1 (Clements et al., 2006). In AD, brains from patients with the disease or with the Lewy body variant exhibit decreased nuclear Nrf2 (Ramsey et al., 2007), implicating an inability of these cells to increase expression of Nrf2 target genes; similar findings have been found in ALS (Kirby et al., 2005). As Nrf2 regulates the expression of a host of genes, these findings encourage further exploration into the regulation of Nrf2 and role of genes under its control.

Maintenance of Glutamate Homeostasis by Astrocytes

Glutamate acts as a major excitatory neurotransmitter in the central nervous system (CNS) and must therefore be carefully regulated at the synaptic cleft. One regulatory mechanism is the glutamate-glutamine cycle. In this process, astrocytes rapidly uptake glutamate from the extracellular space and convert it into glutamine by glutamine synthetase (Norenberg and

Martinez-Hernandez, 1979). Glutamine is released from astrocytes and transferred to nerve terminals where it is taken up into neurons and converted into glutamate by glutaminase. Interestingly, this glutamate can be decarboxylated by glutamic acid decarboxylase (GAD) which will generate GABA, an inhibitory transmitter (Battaglioli and Martin, 1991; Wu et al., 2007). Alternatively, glutamate released into the synaptic cleft can diffuse to post-synaptic elements where it can act on a variety of glutamatergic receptors. The two main classes of these receptors are the ionotropic receptors (NMDA and non-NMDA subtypes) and the metabotropic glutamatergic receptors (mGluR). Ionotropic receptors are predominately involved in rapid signaling, whereas mGluRs are involved in more sustained responses. As the glutamate network relies on fast signaling and most receptors are prone to rapid desensitization, excess extracellular glutamate can have deleterious effects including induction of NMDA-receptor-mediated excitotoxicity which can cause cell death. Though some agents, such as glutamate pyruvate transaminase (GPT), can effectively degrade glutamate (Matthews et al., 2000), studies have indicated that astrocytes act as the primary endogenous regulators of extracellular glutamate levels in the CNS (Rothstein et al., 1996; Peghini et al., 1997; Tanaka et al., 1997). Astrocytes scavenge excess glutamate to prevent neuronal excitotoxic death predominantly through two separate transport systems, the X_{AG-} system comprised of the excitatory amino acid transporters (EAATs1-5) and system x_c-, consisting of the cystine/glutamate antiporter (xCT antiporter).

System X_{AG-}

EAAT1-5, along with the neutral amino acid transporters ASCT1 and 2, are considered members of the soluble carrier gene (SLC) series within the SLC1 family as assigned by the Human Genome Organization Nomenclature Committee. EAAT-1 and EAAT-2 mediate glutamate transport in mature astrocytes, whereas EAAT-3 and EAAT-4 are found predominantly in mature neurons (Palacin et al., 1998; McBean, 2002). Recent work has suggested that there may be levels of EAAT-2 expression in macrophages (Rimaniol et al., 2000). EAAT-5 is primarily located in retina (Arriza et al., 1994; Palacin et al., 1998). Expression of these transporters is developmentally regulated as EAAT-1 and –2 are expressed in immature neurons (Meaney et al., 1998; Yamada et al., 1998) and EAAT-3 and −4 are found in fetal astrocytes (Hu et al., 2003; Maragakis et al., 2003). There is strong evidence to support regional variation with EAAT-1 expression predominately in the cerebellum (Arriza et al., 1994) and high EAAT-2 mRNA expression in astrocytes of the cerebral cortex (Su et al., 2003). EAAT-1 and −3 mRNA is also expressed in non-nervous tissue. EAAT-1 mRNA has high levels of expression in the heart and skeletal muscle, as well as expression in the placenta and lung; EAAT-3 mRNA is in abundance in the kidney, has lower levels in the brain, placenta, lung, and has very low levels in muscle, liver, and heart (Arriza et al., 1994). These expression patterns in human tissue differ from those in rat tissue even though the rat transporters have high homology to their human counterparts (Velaz-Faircloth et al., 1996).

EAATs 1–3 are sodium cotransporters and potassium countertransporters that have a high affinity for L-glutamate (Palacin et al., 1998). Based on mammalian cell assays, EAATs have an affinity for L-glutamate varying from 48 to 97μM (Arriza et al., 1994), though in HeLa cells, oocytes, or rat brain, EAAT-2 has demonstrated higher affinity for glutamate with K_m values between 2 and 18μM (Pines et al., 1992; Arriza et al., 1994; Palacin et al., 1998). These affinity values differ from the millimolar values more typical of amino acid transporters of the epithelium. During transport, one glutamate molecule is coupled with the cotransport of three Na^+ and one H^+ molecules and countertransport of one K^+ molecule with the net result of an inward coupled charged movement of +2 (Zerangue and Kavanaugh, 1996; Levy et al., 1998). These transporters are trimeric proteins consisting of three identical subunits. Though still controversial, crystallographic structures of a glutamate transporter from *Pyrococcus horikoshii*, which has 37% amino acid identity to human EAAT-2, indicate that each individual

subunit has a glutamate binding site (Yernool et al., 2004), supporting the hypothesis of an individual anion pore for each subunit. Recently, sodium-driven glutamate uptake currents have been shown to be independently mediated by each subunit, signifying an active anion conductance even at low glutamate concentrations (Koch et al., 2007).

Expression and localization of EAATs are independently regulated. Epidermal growth factor (EGF), transforming growth factor α (TGF-α), dibutyryl cAMP (dBcAMP), and bromo-cAMP all increased EAAT-2 transcription in human fetal astrocytes (HFA) whereas tumor necrosis factor α (TNFα) decreased expression in HFA (Fine et al., 1996; Su et al., 2003). Recent evidence further suggests that EAAT-2 is highly regulated at the translational level by showing that some EAAT-2 mRNAs have long 5′-untranslated regions (UTR) that, in primary astrocyte cell lines, are translationally regulated by extracellular factors such as corticosterone and retinol (Tian et al., 2007). EAAT-3 localization is highly regulated by neuronal activity (Swanson et al., 1997) and intracellular signaling pathways involving α protein kinase C (α PKC) (Guillet et al., 2005) and phosphatidylinositol-3-kinase (PI3K) (Krizman-Genda et al., 2005) as shown by studies on the rat homolog. Astrocyte-secreted factors have also been shown to affect localization of EAAT-3 (Canolle et al., 2004).

Glutamate-induced neuronal death has been implicated as the cause of neurodegeneration in a host of diseases, with evidence that altered EAAT regulation contributes to the pathology. Neuronal death characterized in HIV-associated dementia (HAD), an infliction that affects 10–20% of HIV-1 infected individuals (Gonzalez-Scarano and Martin-Garcia, 2005), has been linked to glutamate-induced excitotoxicity (Nishida et al., 1996; O'Donnell et al., 2006). When exposed to HIV-related factors *in vitro*, astrocytes exhibit decreased EAAT-2 expression and activity with EAAT-2 mRNA levels decreasing by approximately 66% (Su et al., 2003) and glutamate uptake decreasing by an average of 76% after 6 hours of HIV-1 exposure(Wang et al., 2003). Similar effects on EAAT expression and activity were found in astrocytes exposed to gp120, the HIV-1 envelope glycoprotein, and TNFα, a cytokine released by HIV-infected macrophages and microglia in the CNS (Pitt et al., 2003; Wang et al., 2003). These data suggest that changes in transcriptional regulation of EAAT-2 may contribute to the increases of extracellular glutamate levels seen in HAD. This mechanism is further corroborated by evidence that interruption of proper glutamate regulation following treatment with gp120 causes memory impairments (Fernandes et al., 2006), a common clinical symptom in HAD. As the neurodegeneration characterized in HAD is an indirect effect of HIV-infected macrophages and microglia that have an activated inflammatory response, it is possible that the changes in glutamate receptors associated with HAD will be translatable to other neuroinflammatory conditions.

Aberrant EAAT expression has been implicated in other neurodegenerative diseases. Knocking out the mouse homolog of EAAT-2 leads to lethal spontaneous seizures, neurodegeneration in the hippocampus, and increased susceptibility to acute cortical injury (Tanaka et al., 1997). Specifically, Tanaka et al (1997) found that knockout mice had spontaneous seizures that resembled NMDA-induced seizures and premature death. In concordance with these findings, the sporadic form of ALS, characterized by loss of motor neurons (Wisman et al., 2003) and by increased glutamate levels in cerebrospinal fluid (CSF) (Spreux-Varoquaux et al., 2002), has up to 95% reduction in EAAT-2 expression in motor cortex and spinal cord (Howland et al., 2002). One hypothesis is that this EAAT reduction is due to RNA processing (Lin et al., 1998), but more recent evidence implicates translation attenuation (Tian et al., 2007), a state that can be induced by ISR as well. In AD, the hippocampus and gyrus frontalis medialis have decreased EAAT-2 expression, especially around the amyloid plaques, whereas the cerebellum has upregulated EAAT expression (Jacob et al., 2007). However, the transgenic APP/Ld/2 mouse, which overexpresses a 695 amino acid form of human amyloid precursor protein (APP),

exhibits a decrease in glial-specific glutamate transporter activity that corresponds with decreased protein expression but not mRNA levels (Masliah et al., 2000).

Neuronal EAAT-3 has been similarly studied in seizures as well as in transient ischemia. In patients with temporal lobe epilepsy with hippocampal sclerosis, though there were fewer total EAAT-3 positive cells, EAAT-3 expression was increased in individual neurons in CA2 and in the granule cell layer of the dentate gyrus compared with patients without hippocampal sclerosis (Mathern et al., 1999; Proper et al., 2002). Studies have also looked at EAAT-3 regulation after short ischemic events that act as a form of preconditioning, resulting in resistance to damage after subsequent severe ischemic injury. After induction of a short cerebral ischemic event in rats, there is neuronal upregulation of EAAT-3 with particular intensity at the plasma membrane; this upregulation appears to be regulated by TNFα and the TNFα receptor subfamily member 1 (TNFR1) (Pradillo et al., 2006). Though disease or injury to the CNS seems to affect EAAT-2 and EAAT-3 expression similarly, in contrast to EAAT-2, EAAT-3 does not demonstrate changes in the frontal cortex of AD patients by immunoreactivity or at the mRNA level (Li et al., 1997). As damage to the frontal cortex underlies the symptomatology of AD, in regards to glutamate transport, it is the affects of EAAT-2 regulation that might be important in the neuropathogenesis of this disease.

As the structure and kinetics of EAATs continue to be elucidated and their role in disease explored, there has been a great push to discover pharmacological agents that alter function of these glutamate transporters with a goal of developing therapeutics (reviewed in (Bridges and Esslinger, 2005)). Most inhibitors to date that show selectivity are limited to EAAT-2 inhibitors (Vandenberg et al., 1997; Eliasof et al., 2001; Shimamoto et al., 2004; Moussa et al., 2007). However, as the structural differences between the individual EAATs are further elucidated, it may be easier to exploit these differences to increase selectivity. More importantly, understanding the structure of these receptors may aid in the development of functional enhancers which may be needed to compensate for the lost EAAT activity observed in neurodegenerative diseases.

System x_c

The other major glutamate transport system in astrocytes, the x_c- system, also referred to as the cystine/glutamate transporter and as the xCT antiporter, mediates over 50% of glutamate transport in glioma cell lines (Ye et al., 1999). It acts as an antiporter by taking up cystine in exchange for glutamate export. By blocking glutamate release from this antiporter, the rat striatum has a 60% decrease in extrasynaptic glutamate levels, implicating that the origin of this glutamate is from non-vesicular release, and that system x_c- is a major source (Baker et al., 2002). Studies on cocaine withdrawal in rats have also implicated system x_c- as an important regulator of extracellular glutamate (Baker et al., 2003). System x_c- also has a vital role in cellular protection against oxidative stress (Lewerenz et al., 2006; Shih et al., 2006), as the import of cystine is vital to the synthesis of glutathione. As the antiporter plays an essential for glutathione production in exchange for increasing extracellular glutamate, its regulation must be carefully controlled. We believe that aberrant expression and/or activity of system x_c- may contribute to excitotoxicity during neurodegeneration

The x_c- system is a highly regulated electroneutral system that exchanges cystine for glutamate in a Na^+-independent 1:1 ratio (Palacin et al., 1998; Sato et al., 1999). The antiporter is composed of two subunits: a heavy chain comprised of a cell surface antigen 4F2hc that is necessary for antiporter translocation to the plasma membrane (Bassi et al., 2001), and a light chain known as xCT that has two variants formed by alternative splicing (McBean, 2002), either of which can confer substrate specificity. In humans, the xCT light chain has been termed SLC7A11 by the Human Genome Organization Nomenclature Committee. In mice, xCT is a 502 amino acid protein with 12 assumed transmembrane domains (Sato et al., 1999; Gasol et

al., 2004). Though the predicted size of the xCT protein is approximately 55kDa (Sato et al., 1999), research in mice has found that an xCT antiserum recognizes 40kDa and 80kDa bands that correspond to the xCT protein (Burdo et al., 2006; La Bella et al., 2007). Northern blot analysis has revealed xCT mRNA in mouse brain, rat cortical cells, retinal ganglion cells, and human glioma cells (Sato et al., 2002; Burdo et al., 2006; Dun et al., 2006), but not in mouse heart, kidney, liver, or lung (Sato et al., 1999). Immunocytochemical studies in rat brain have found strong xCT expression in all cell types examined, with increasing expression throughout development (La Bella et al., 2007). In the adult mouse brain, xCT mRNA has robust expression in areas facing the cerebral ventricles, medial habenuclei, and in the meninges, scattered expression in the hypothalamic regions, and low level expression in the cortex and cerebellum (Sato et al., 2002).

Within the 5′-flanking region of the xCT gene are several sequences resembling the *cis*-acting electrophilic response element (EpRE), also known as the antioxidant response element (ARE), that is responsible for the induction of the adjacent genes. As such, xCT is transcriptionally regulated by electrophilic agents such as diethylmaleate, and hydroquione by activation of the endogenous antioxidant response via the Nrf2 transcription factor (Sasaki et al., 2002). During oxidative stress or other events that cause the activation of the cellular integrated stress response (Cullinan et al., 2003), Nrf2 translocates to the nucleus where, in the presence of small Maf proteins, it binds the ARE and up-regulates transcription of these ARE-containing genes. Nrf2 has been found to regulate the xCT antiporter in astrocytes (Qiang et al., 2004), with overexpression of Nrf2 in rat cortical cultures leading to increased xCT mRNA expression, xCT activity, and intracellular glutathione levels (Shih et al., 2003). Though Nrf2 has been found to control the glutathione pathway (Sun et al., 2005), Nrf2 is not the only regulator of xCT, though other methods of regulation have yet to be determined (Pacchioni et al., 2007). One recently suggested mechanism is through the ISR-regulated transcription factor, ATF4, which seems to be important in controlling xCT expression during amino acid deprivation (Sato et al., 2004). As neurodegenerative conditions can induce oxidative stress, upregulation of the antioxidant response, including the xCT antiporter, can act as a protective mechanism by preventing damage from oxidative stress. In HIV-associated dementia (HAD), studies have found the exposure of retinal pigment epithelial to the transactivator protein, TAT, induces xCT expression (Bridges et al., 2004). As the most abundant source of oxidative stress in the brain is from the respiratory burst of activated microglia (Colton and Gilbert, 1987; Colton et al., 2000), a major pathological hallmark in HAD, it is not surprising to see changes in the antioxidant pathway and, therefore, in xCT in this disease.

Though important in glutathione synthesis, upregulation of the xCT antiporter can have potentially damaging effects, as it is a mechanism for glutamate export and critical in the regulation of extracellular glutamate (Augustin et al., 2007). The pathology behind a variety of neurological insults takes advantage of the xCT antiporter to propagate cell damage. In glial-derived tumors, or gliomas, high levels of glutamate are released, causing excitotoxic neuronal death, mediated by increased xCT expression (Chung et al., 2005). When pharmacological inhibitors are used to block system x_c^- activity, the progression of tumor growth is disrupted (Chung et al., 2005). Kaposi's sarcoma-associated herpesvirus (KSHV), a virus linked to lymphoproliferative syndromes that frequently affects immunocompromised individuals, also uses the xCT antiporter, as KSHV cell fusion and virion entry correlates closely with expression of the xCT light chain of the antiporter (Kaleeba and Berger, 2006). In AD, inhibition of system x_c^- may also prove advantageous for neurons. In neuronal cultures exposed to microglia, exposure to amyloid β-peptides (Aβ) increases microglial xCT mRNA expression and glutamate release which can trigger neuronal death; if system x_c^- is inhibited, microglia can increase release of apolipoprotein E rescuing neurons from Aβ toxicity (Qin et al., 2006), indicating a potential deleterious role of system x_c^-. Under these situations, attenuating xCT antiporter activity would be expected to have beneficial outcomes. Thus, regulation of system

x_c- requires a delicate balance, ensuring adequate cystine for glutathione synthesis, but minimizing glutamate export.

Regulation of Glutathione by Astrocytes

Oxidative stress can lead to endoplasmic reticulum (ER) stress as the ER attempts to handle the accumulation of oxidized proteins. Cystine is one of the required components in the synthesis of glutathione (GSH), an important antioxidant of the CNS. In exchange for glutamate, cystine is transported into the cell through the xCT antiporter. Once in the cell, cystine is reduced to cysteine. A reaction between cysteine and glutamic acid results in γ-glutamyl-cysteine, which in turns reacts with glycine to form GSH (Peuchen et al., 1997). As cysteine acts as the rate limiting element in GSH synthesis, the xCT antiporter is crucial for maintaining GSH homeostasis (Dun et al., 2006), and is critically important in protecting cells from free radicals, peroxides, and other oxidizing agents (O'Connor et al., 1995).

Glutathione is heterogeneously distributed throughout the nervous system, potentially affecting region-and cell-specific capacities to respond to oxidative stress (Philbert et al., 1991). In the central nervous system, GSH is compartmentalized between glia and neurons, with glial cells exhibiting higher levels of GSH (Knollema et al., 1996), especially microglia (Chatterjee et al., 1999). GSH released from astrocytes provides the precursors for neuronal GSH synthesis. GSH acts as an antioxidant by either catalyzing the nucleophilic addition of the sulfur atom in glutathione to an electrophilic group, or by covalently binding with reactive metabolites (Ahlgren-Beckendorf et al., 1999), upon which it is oxidized to glutathione disulfide (GSSG). Therefore, not only is GSH an essential antioxidant, it also participates in a variety of cell processes such as cell proliferation and regulation of apoptosis, detoxification of xenobiotics, thiol redox state, and storage and transport of cysteine, among others (reviewed in ((Cotgreave and Gerdes, 1998; Arrigo, 1999; Dringen, 2000)).

Altered glutathione levels are characterized in a variety of conditions from PD (Sofic et al., 1992) to schizophrenia (Yao et al., 2006) as well as in normal aging (Sohal and Weindruch, 1996). In HIV patients, glutathione levels are altered by viral proteins (Price et al., 2005) and act as a key determinant of patient survival, with decreased GSH associated with a markedly decreased survival rate (Herzenberg et al., 1997); administration of the GSH pro-drug N-acetylcysteine (NAC) replenishes GSH, identifying itself as an important potential therapeutic (De Rosa et al., 2000; Muller et al., 2000). Targeting the consequences of glutathione depletion has proved an effective method to attenuate neuronal loss in models of CNS disorders (Malorni et al., 1998; Anderson et al., 2004; Hart et al., 2004).

As more glutathione is needed, logic stands that xCT activity and/or expression would need to be augmented in order to increase glutathione synthesis. However, as upregulation of this antiporter mediates the regulation of extracellular glutamate (Augustin et al., 2007), careful regulation of system x_c- is needed in order to maintain a balance between protective glutathione synthesis and toxic increases in extracellular glutamate.

Neuronal Responses to Altered Astrocyte Function

NMDA Receptor Stimulation

Increased release of glutamate from astrocytes mediates their neurotoxic activities through the NMDA receptors. Functional NMDA receptors are heteromeric assemblies of four subunits: two NMDA-R1 subunits and two NMDA-R2. Together, the four subunits form a channel in the plasma membrane permitting influx of $^{Ca2+}$ when stimulated. The NMDA-R1 subunits (NR1) are derived from a single gene that can exist as eight different variants based on alternative splicing (Goebel et al., 2005), while four separate genes encode the NMDA-R2 subunits (NR2A, NR2B, NR2C and NR2D) (Lynch and Guttmann, 2001; Waxman and Lynch,

2005). The NR1 subunit binds glycine, while the NR2 subunit binds glutamate and quinolinic acid. Although all four NR2 subunits bind glutamate with equal affinity, NR2A and NR2B trigger greater excitotoxicity than NR2C and NR2D (reviewed in (Lynch and Guttmann, 2001; Waxman and Lynch, 2005). Quinolinic acid activates NR2A- and NR2B-containing receptors, but not those containing NR2C or NR2D subunits (Carvalho et al., 1996). NR2B-containing NMDA receptors are often located extrasynaptically (Charton et al., 1999; Ali and Salter, 2001; Riccio and Ginty, 2002). Interestingly, brain regions enriched in NMDA receptors composed of NR2A and NR2B subunits, which include the hippocampus, striatum, forebrain, are commonly injured in excitotoxic insults such as ischemia, epilepsy and also in HIV-1 infection (Conti et al., 1999; Everall et al., 1999; Heyes et al., 2001; Lynch and Guttmann, 2002; Archibald et al., 2004; Sa et al., 2004; Waxman and Lynch, 2005). Furthermore, regions such as the cerebellum that express relatively high levels of NR2C and low levels of NR2B are commonly spared in excitotoxic insults (Lynch and Guttmann, 2002).

There are two inter-related levels of regulation of NMDA receptor expression and function that ultimately influence their roles in neuronal survival and death: NR subunit expression by developmental stage and phosphorylation state. A major mechanism of regulation of the NMDA receptor is the phosphorylation/de-phosphorylation by neuronal kinases and phosphatases. The phosphorylation state of NR1 and NR2 NMDA receptor subunits regulates their function and can also influence their localization to synaptic and extra-synaptic sites by regulating associations with cellular proteins. NR1 subunits are typically phosphorylated on serine and threonine residues (ser896, ser897) while NR2A and NR2B are typically phosphorylated on tyrosine residues (tyr842, tyr1387, tyr1472), as well as at other residues (Kennedy and Manzerra, 2001; Li et al., 2001; Chung et al., 2004; Besshoh et al., 2005). NMDA receptor function is modulated by tyrosine kinases and phosphatases, as well as serine/threonine kinases, although how this modulation is achieved is not clear (Kennedy and Manzerra, 2001; Li et al., 2001; Goebel et al., 2005). In NR1, phosphorylation of ser896 and ser897 is required for PKC-mediated release of NR1-containing NMDA receptors from the ER and may play a role in NMDA receptor membrane trafficking (Scott et al., 2001). Brain-derived neurotrophic factor (BDNF) causes a rapid phosphorylation of NR1 that is associated with potentiation of synaptic transmission (Suen et al., 1997).

In NR2 subunits, the C-terminal tails of both NR2A and NR2B contain three tyrosines located within YXXØ motifs (NR2A: tyr 842, tyr1325, tyr 1454, and NR2B tyr843, tyr1336, and tyr1472). Phosphorylation of tyr1472 in NR2B located within the YXXØ motif correlates with amyloid-β-induced internalization of the NMDA receptor in cortical neurons (Snyder et al., 2005), while inhibition of tyr842 phosphorylation on NR2A is associated with decreased membrane expression (Vissel et al., 2001). Notably, ischemic insult to neurons is associated with increased NR1 (ser896), NR2A (tyr1387) and NR2B phosphorylation, as well as with enhanced association of these receptors within the postsynaptic density (Cheung et al., 2003; Besshoh et al., 2005). The net effect is the increased synaptic expression of NR2A– and NR2B-containing NMDA receptors. However, loss of tyr1472 phosphorylation in NR2B has been associated with stabilization of NR2B on the cell surface, as well as with increased internalization through endocytosis via clathrin coated pits (Roche et al., 2001; Snyder et al., 2005). It thus remains to be determined how tyr1472 phosphorylation affects hippocampal neuronal sensitivity to excitotoxicity. Recently, cdk5-dependent phosphorylation of NR2A ser1232 *in vitro* and *in vivo* has been shown to regulate NMDA-induced long-term potentiation (LTP) in CA1 hippocampal neurons (Kennedy and Manzerra, 2001; Li et al., 2001). Furthermore, enhanced phosphorylation of NR2B tyr1336 might be associated with activation of neuronal survival pathways (Wu et al. J. Biol. Chem. 13: 20075–20087).

Another important consideration regarding the role of NR2 subunits in excitotoxic death is their site of localization. Generally, extrasynaptic NMDA receptors primarily containing

NR2B subunits are thought to mediate excitotoxicity, whereas synaptically-associated NMDA receptors mainly containing NR2A subunits at ages when both subunits are expressed, are associated with neuroprotective functions, synaptic plasticity, and long-term potentiation (Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999; Sattler and Tymianski, 2001; Hardingham and Bading, 2003). Synaptic and extrasynaptic localization of NMDA receptors are differentially regulated by their phosphorylation state (Li et al., 2002), and by their association with post-synaptic density proteins (PSD-95, SAP 102) (Dong et al., 2004). Thus, specific phosphorylation events within the C-terminal tails of NR subunits provide a major level of regulation of neuronal entry into both survival and death pathways.

A second major level of regulation of NMDA receptor function is the developmentally-linked expression of NR subunits (Liu et al. J. Neuroscience 27: 2846–2857). NR2A and NR2B-containing receptors demonstrate varying temporal expression in the rat hippocampus and the expression coincides with hippocampal neuronal susceptibility to age-dependent excitotoxicity in maturing cultures (O'Donnell et al., 2006); mouse cortical cultures demonstrate a similar age-dependent effect in regards to glutamate toxicity (Fogal et al., 2005). These observations suggest a common pathway of neuronal death mediated by NR2A and/or NR2B subunits in classic excitotoxic brain injury, including HIV-1 infection. Accordingly, the development of NR2A- (Siao and Tsirka, 2002) and NR2B-selective antagonists (Lynch and Guttmann, 2001) has been driven by a widespread interest in producing non-toxic, selective NMDA receptor antagonists as neuroprotectants against such brain injury (Lipton, 2004). Most NMDA receptor-mediated toxicity is regulated through calpain activation, hence, selective NR subunit inhibitors and calpain antagonists can provide neuroprotection.

Activation of Calpains in Neurons

Excitotoxicity due to overstimulation of NMDA receptors results in increased cytosolic calcium concentrations ($[Ca^{2+}]$). A family of calcium-activated cysteine proteases, the calpains, has been implicated in modulating cellular mechanisms in response to changes in $[Ca^{2+}]$ (Guroff, 1964; Wang et al., 1992). Calpains are upregulated in a variety of neurodegenerative conditions, both acute, such as traumatic brain injury (McCracken et al., 1999) and brain ischemia (Yamashima, 2000; Blomgren et al., 2001; Shioda et al., 2006), and chronic, such as multiple sclerosis (Shields et al., 1999), AD (Grynspan et al., 1997; Tsuji et al., 1998; Raynaud and Marcilhac, 2006), Huntington's disease (Lee et al., 2006), and HAD (Vanderklish and Bahr, 2000; Ray and Banik, 2003; Wang et al., 2007). Calpains are thought mainly to function intracellularly and have been found in virtually all vertebrate cells, although calpains or calpain-like genes have been identified in Drosophila and other invertebrates (Goll et al., 2003). There are 14 identified human genes in the mammalian calpain family encoding the 80kDa cysteine protease domains and two encoding the small 30kDa regulatory subunit domain (Suzuki et al., 2004). The mammalian calpain genes share over 90% homology and there is little evidence of alternative splicing events (Suzuki, 1990). Though calpain substrate selectivity is not completely understood and may depend on the target peptide's tertiary structure, some substrates can be distinguished by a sequence rich in proline, glutamate, and serine/threonine residues (Rechsteiner and Rogers, 1996; Tompa et al., 2004).

Two calpain isoforms predominate, μ–calpain and m-calpain (Croall and DeMartino, 1991). Both isoforms share substrate specificities (Molinari and Carafoli, 1997) and are heterodimers composed of a regulatory 28 kDa subunit that is identical between the two isoforms and an 80-kDa subunit that shares 55–65% sequence homology (Goll et al., 2003). Although μ–calpains have been described more prevalently in neurons and m-calpains in glia (Hamakubo et al., 1986), they are classically distinguished by the *in vitro* $[Ca^{2+}]$ requirement for their half maximal activation: μ–calpain (3–50 μM) and m-calpain (400–800 μM) (Suzuki, 1991; Ray et al., 1999; Goll et al., 2003). Since physiologic $[Ca^{2+}]$ generally range from 50–300nM, and

[Ca^{2+}] necessary to activate m-calpain would result in immediate cell death regardless of calpain activation, calpain must utilize mechanisms that reduce their required [Ca^{2+}]. In the presence of Ca^{2+}, μ–calpains and m-calpains both undergo autoproteolysis that reduces their [Ca^{2+}] activation requirement by over half (Saido et al., 1994; Goll et al., 2003). In addition, phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP_2), can also further reduce the [Ca^{2+}] activation requirement (Saido et al., 1992). Phosphorylation of the calpains may also play a role in determining their activation (Goll et al., 2003). ERK phosphorylation of Ser50 of m-calpain can reduce its activation threshold to under 1μM Ca^{2+} (Glading et al., 2002). These steps may be necessary for calpain activation under physiologic conditions.

Once activated, calpains are known to regulate multiple cellular mechanisms through cleavage of their substrates. The limited number of calpain cleavage sites on a given peptide often results in large, catalytically active fragments and suggests that the role of calpains is often regulatory, as opposed to the degradational roles of proteases in lysomes or proteasomes (Goll et al., 2003; Franco and Huttenlocher, 2005). Along these lines, calpains have been implicated in long-term potentiation in the hippocampus via direct or indirect modulation of kinases, glutamate receptors, or receptor interacting proteins (Tomimatsu et al., 2002). Further alteration of cellular activity may occur due to proteolytic modulation of signal transduction (Kishimoto et al., 1989; Pariat et al., 1997; Pfaff et al., 1999; Pariat et al., 2000), gene expression and transcription factors, cell cycle machinery (Kubbutat and Vousden, 1997; Zhang et al., 1997), and apoptosis (Squier et al., 1999; Chua et al., 2000; Lu et al., 2002).

Both apoptotic- and necrotic-like death have been described following excitotoxic stimulation depending on the stress severity (Lieberthal et al., 1998). Calpains have a variety of roles in regulating apoptotic mechanisms in cells, including prevention of apoptosome formation. This action prevents the induction of the intrinsic mitochondrial apoptotic pathway despite potential mitochondrial cytochrome *c* release and, therefore, may promote a more necrotic-like mechanism of death (Lankiewicz et al., 2000). Activation of μ-calpains has also been shown to lead to lysosomal membrane degradation and, thus, release of cathespin B and L (Yamashima, 2000). Cathepsin could contribute to breakdown of cellular components and this has been demonstrated in hippocampal neurons (Kitao et al., 2001). However, calpains can also have pro-apoptotic effects due to their cleavage of Bcl-2 family proteins (Gil-Parrado et al., 2002), apoptosis-inducing factor (Polster et al., 2005), Bax (Wood et al., 1998; Gao and Dou, 2000; Hwang et al., 2003), and Bid (Chen et al., 2001; Chen et al., 2002; Mandic et al., 2002; Takano et al., 2005; White et al., 2007). Furthermore, calpains can both directly activate and deactivate caspases through cleavage (Chua et al., 2000; Nakagawa and Yuan, 2000).

Calpains play an important role in disassembling adhesion and cytoskeletal components at the plasma membrane, which are essential for the mechanisms of cell motility (Glading et al., 2002; Perrin and Huttenlocher, 2002). Because of this, calpains may play important roles in neurite outgrowth or remodeling in neurons (Howard et al., 1999). Along these lines, some of the major calpain substrates are adhesion molecules and cytoskeletal proteins, including vimentin, spectrin, tau, actin, tubulin, glial fibrilliary acidic protein, neurofilaments, and microtubule-associated proteins. Activation of calpains during synaptic activity is also crucial for neurotransmitter release, synaptic plasticity, vesicular trafficking, and structural stabilization (Wu and Lynch, 2006). Activation of calpains due to excitotoxic insult can result in synaptic dysfunction or inappropriate disassembly of cellular structures and may contribute to the classic morphologic impact seen following excitotoxic stress (Buki et al., 2003; O'Hanlon et al., 2003). As such, calpains have been implicated in Wallerian degeneration (Zhai et al., 2003), acute axonal degeneration (Kerschensteiner et al., 2005), and in the loss of soma, neurites, and synapses in aged primary neuronal cultures (Kim et al., 2007).

Calpains may further exacerbate excitotoxic conditions by cleaving proteins critical to cytosolic [Ca^{2+}] regulations, such as the plasmalemmal Ca^{2+} ATPase (Guerini et al., 2003). μ-calpains are thought to be closely linked to NMDA receptor function (Hewitt et al., 1998). In fact, its ability to cleave the NR2 subunits (N-terminal of NR2A, NR2B, and NR2C and C-terminal of NR2A) of the NMDA receptor may lead to modulation of excitotoxic conditions (Bi et al., 1998; Guttmann et al., 2001; Simpkins et al., 2003). Electrophysiological properties of the NMDA receptors are not altered by these cleavages, but these cleavages may regulate localization, turnover, or signaling. Along these lines, the C-terminus of NR2 subunits in the cytoplasmic side of the cellular membrane interact with various kinases and synaptic proteins, such as PSD-95, which is involved in vesicle trafficking. Guttmann et al (2001) have speculated, and transgenic studies have suggested (Sprengel et al., 1998), that cleavage of the NR2 subunit results in NMDA receptors that are prevented from interacting with cytosolic signaling pathways. In addition, it has been suggested that calpain-cleaved NR2B-containing NMDA receptors may linger on the surface of neurons and thus increase neuronal excitotoxic susceptibility (O'Donnell et al., 2006).

Altered cell cycle regulation is another means by which excitoxic calpain activation could induce neuronal dysfunction. Aberrant activation of cell cycle machinery has been linked to a variety of neurodegenerative diseases (reviewed in (Herrup and Yang, 2007)). As suggested by Carragher et al, calpain activity may modulate progression through the G1 phase of the cell cycle, and increased expression of the endogenous calpain inhibitor calpastatin correlates with decreased cyclin A, D, and cdk2, as well as decreased levels of retinoblastoma protein phosphorylation (Carragher et al., 2002). Calpains have also been implicated in the regulation of the E2F1 transcription factor, which is known to regulate proliferation and neuronal death (Strachan et al., 2005).

Other calpain substrates whose cleavage could alter neuronal function include ion channels and transporters, growth factor receptors, phospholipases, kinases and phosphatases (Goll et al., 2003). Since calpains themselves can be phosphorylated, their activation may have autoregulatory roles. Additionally, calpains may alter neuronal function by modifying kinase and signal transduction pathways in neurons through cyclin dependent kinase 5 (CDK5) activation. Calpain cleavage of p35 to its more stable isoform, p25, increases the level, activity, and subcellular distribution of the p25:CDK5 complex (Musa et al., 1998; O'Hare et al., 2005). This complex has been associated with CDK5 neurotoxic function (Patrick et al., 1999), and pharmacologic inhibition of calpains and CDK5 has shown to be protective in some excitotoxicity models (Wang et al., 2007).

The most stringently specific calpain inhibitor is the endogenously expressed calpastatin, which is a major mediator of calpains and is, itself, dependent on Ca^{2+} for its inhibition of calpains (Tullio et al., 1999; Goll et al., 2003). Synthetic calpain inhibitors, on the other hand, lack the exact calpain specificity of calpastatin, but do successfully protect neurons from excitotoxic stress. Calpain inhibitors PD 150606 and MDL-28170 can reduce degeneration in cultured neurons following excitotoxic or other stress stimuli (Wang et al., 2007). MDL-28170 is neuroprotective in both *in vitro* and *in vivo* models of traumatic brain injury (Wang and Yuen, 1997; Laurer and McIntosh, 2001) and several calpain inhibitors have shown significant neuroprotection in animal models of CNS injury and disease (Ray and Banik, 2003). However, clinical trials involving calpain inhibitors have thus far shown little promise (Wang et al., 2006).

Because calpains have seemingly contradictory roles in preventing and initiating apoptotic processes, as well as in regulating critical cellular functions, it is hard to define their role as simply neurodegenerative or neuroprotective. Indeed, the system that may be responsible for the blebbing and disintegration of neuronal processes might also serve a critical role in

remodeling cytoskeletal proteins important for the neurite regrowth that is seen following sublethal excitotoxic insults. Most likely, the role of calpains is dependent on both the type and severity of the stress (Moore et al., 2002). Likewise, the function of calpain activation could be site-specific within a cell, as non-homogeneous distribution of [Ca^{2+}] can locally activate calpains (Valeyev et al., 2006). Alternatively, the effect of calpains could be dependent on the isoform composition of the local calpain pool. The resultant magnitude, duration, and localization of cytosolic [Ca^{2+}] following an excitotoxic insult could all be critical determinants of the role calpains will play in excitotoxic responses.

Summary and Model

Taken together, the pathways described in this review have known physiologic roles in CNS function and response to stress. Certainly, astrocytes need to activate Nrf2 to produce glutathione and reduce oxidative stress. However, we propose that overstimulation of protective pathways, such as the antioxidant response and the integrated stress response, in astrocytes during chronic, progressive exposure to neurodegenerative stimuli will result in neuronal damage and death by altering the balance of glutamate transport. In our model (Figure 2), activation of the ISR in astrocytes will increase glutamate export by the cystine/glutamate antiporter to increase import of cystine for glutathione production. At the same time, inflammatory mediators such as TNFα or the ISR via altered ER and golgi processing will repress EAAT-1 and EAAT-2 expression and activity removal of excess glutamate from the extracellular milieu. Such changes will alter astrocytic glutamate homeostasis leading to net extracellular accumulation of glutamate. Failure of the astrocytes to regulate glutamate will result in stimulation of neuronal NMDA receptors, perhaps extra-synaptically, leading to Ca^{+2} release and activation of calpains. Initially, activation of calpains may have limited local effects on dendritic morphology, but overtime, its activation may lead to neuronal death. We propose that the initial local changes in dendritic morphology would cause neuronal dysfunction, which would manifest clinically, but would be reversible if treated. However, chronic, progressive activation of calpains would ultimately culminate in neuronal loss making the clinical deficits fixed. These studies suggest several therapeutic targets for patients with neurodegenerative diseases, but also highlight the complexity of treating astrocytic dysfunction, as reducing effects of glutamate toxicity may reduce antioxidant activity, thus trading one mechanism of cellular toxicity for another. As we expand our understanding of the regulatory mechanisms governing astrocytic and neuronal responses to stress, comprehensive treatment programs for patients with neurodegenerative diseases will emerge.

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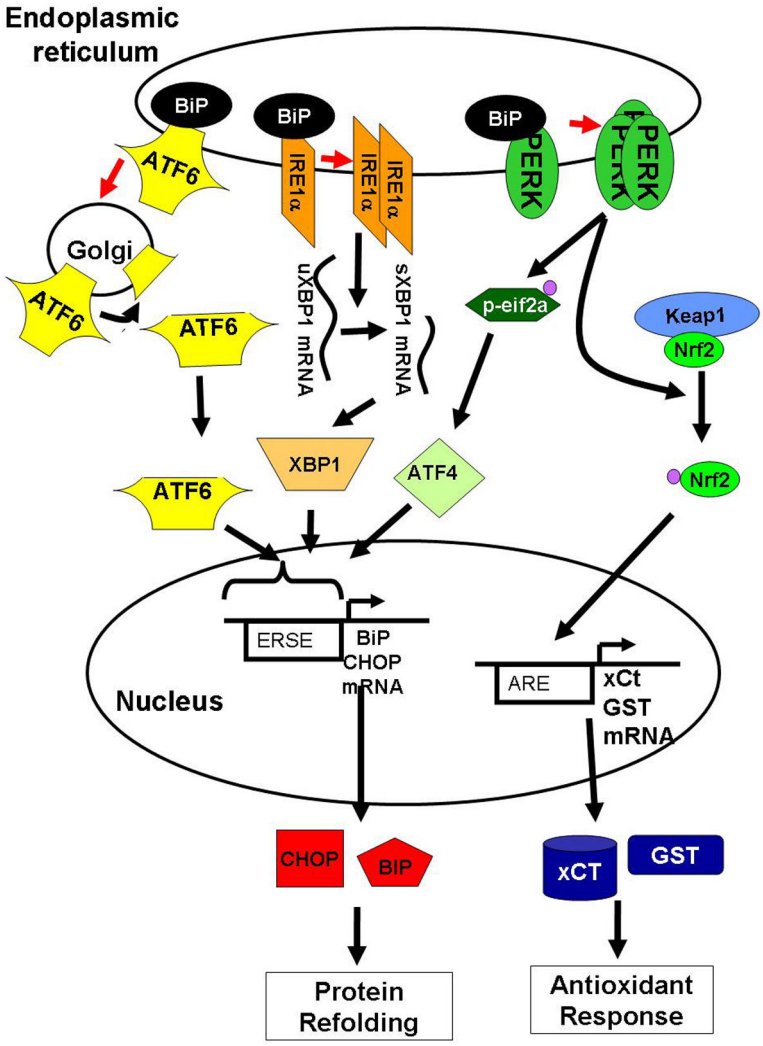

Figure 1. The integrated stress response (ISR)

In response to oxidative stress, unfolded proteins, inflammation and other cellular stresses, a stress response is activated to reduce cellular metabolism until cellular homeostasis is restored. In response to stress, the endoplasmic reticulum chaperone protein BiP utilization by unfolded proteins frees the 3 initiators of the ISR: ATF6, IRE1α, and PERK. ATF6 is translocated to the golgi and cleaved releasing ATF6-p50, a transcription factor. Free IRE1α splices uXBP1 to sXBP1 which can now be translated. PERK phosphorylates 2 known substrates, eIF2α and Nrf2. eIF2α depresses general translation initiation, but favors translation of ATF4. ATF6-p53, sXBP1, and ATF4 translocate to the nucleus and activate transcription of chaperone proteins like BiP to re-establish homeostasis. Concomitantly, phosphorylation of Nrf2 by PERK disrupts interaction between Keap1 and Nrf2 leading to translocation of Nrf2 to the nucleus and transactivation of antioxidant genes including GST and xCT. Together, these responses reduce oxidative stress and establish protein refolding.

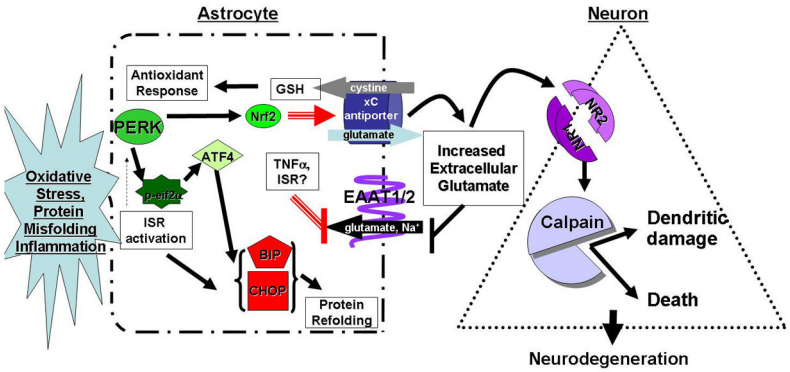

Figure 2. Model for ISR activation in altering astrocyte neuroprotection

In neurodegeneration, ISR activation occurs due to chronic exposure to reactive oxygen species, unfolded proteins and/or inflammatory mediators. Chronic activation of ISR leads to constant elevation of the glutamate/cystine antiporter (xCT) via transcriptional activation by Nrf2. Increased xCT exports glutamate out of the astrocyte in order to import cystine for glutathione generation to reduce reactive oxygen species. Inflammatory mediators and, potentially, ISR activation results in decreased EAAT1/2 activity blocking glutamate uptake from the extracellular milieu, leading to an increase in extracellular glutamate. Increased extracellular glutamate is free to stimulate NMDA receptors on neurons causing influx of Ca^{2+} ions. Increased Ca^{2+} levels activate calpain proteases causing damage to dendritic spines and dendrites. Such damage to neurons will lead to neuronal dysfunction. Finally, chronic or extreme activation of calpain will lead to cell death.