ALTERATIONS IN CELLULAR GLUTAMATE TRANSPORT DO NOT CONTRIBUTE TO NEURONAL CELL DEATH IN A MIXED CORTICAL CELL CULTURE MODEL OF HYPOGLYCEMIA

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Abstract:

Severe hypoglycemia is associated with neurological deficits that when left untreated can lead to frank neuronal cell death. Despite longstanding evidence in both *in vitro* and *in vivo* models that hypoglycemic neuronal cell death is mediated by glutamate excitotoxicity, the cellular and molecular mechanisms involved remain incompletely defined. Toward this end, we recently reported that glutamate efflux from astrocytes via the anionic cystine/glutamate antiporter, system x_c^-, contributed to glucose-deprivation (GD) induced neuronal cell death *in vitro.* However, the precise mechanism by which system x_c^- activity links to glutamate-mediated injury has yet to be determined. Thus, the overall purpose of this thesis was to investigate whether changes in system x_c^- expression in our astrocyte and mixed cortical cell cultures and/or alterations in glutamate handling in a mixed cortical culture model following glucose deprivation occur(s). Toward the former, no change in the expression of mRNA (GD up to 4 h) or protein (GD up to 8 h) of xCT, the functional light chain of system x_c^-, in either astrocyte or mixed cortical cell cultures was demonstrated via quantitative RT-PCR or western blot analysis, respectively. Further, aglycemic neuronal injury, induced by 6 or 8 h of glucose deprivation, was not prevented by the addition of either actinomycin D (10 μg/mL) or cycloheximide (1 μg/mL), demonstrating no requirement for transcription or translation, respectively. Toward the latter, alterations in classical glutamate re-uptake transporter function also did not appear to be altered. Media containing added glutamate taken from control astrocytes or astrocytes deprived of glucose (6 h) was equally toxic to pure neuronal cultures, demonstrating no alterations in glutamate removal between control and glucose-deprived cells. However, neurons in mixed cortical cell cultures deprived of

glucose showed increased neuronal cell death over those maintained in glucose-containing medium when exposed directly to equimolar concentrations of either glutamate or NMDA. Similarly, this increased neuronal death in glucose deprived mixed cortical cultures was shown across several different time points using constant concentrations of either glutamate or NMDA. Lastly, we show that neurons in our mixed cortical cultures are fully protected from excitotoxic cell death when system x_c^- and NMDA receptor inhibitors are added up to two hours following the initiation of glucose deprivation. Overall, our data reveal that neither enhanced system x_c^- expression nor impaired glutamate uptake could account for the neuronal cell death induced by glucose deprivation, but that energy deprived neurons appear simply more susceptible to excitotoxic insults. Therefore, physiological levels of glutamate released from astrocyte system x_c^- may be sufficient to mediate neuronal cell death under aglycemic conditions.

Introduction:

Hypoglycemia

Blood glucose concentrations range between 3.9 and 7.1 mM (Gruetter et al., 1998). Hence, hypoglycemia is defined as a blood glucose concentration below 3.9 mM. In the case of severe hypoglycemia, blood glucose concentrations fall below 2.0 mM. Hypoglycemia is considered one of the most important complications in patients attempting to tightly regulate glucose levels with insulin and is the limiting factor in the development of strategies that aim to maintain normoglycemia in diabetic patients (Lincoln et al., 1996, Lacherade et al., 2009). Hypoglycemia is also a complication in neonates and patients with insulin-producing tumors (Anderson et al., 1967, de Herder, 2004).

As the human brain consumes approximately 25% of the circulating glucose (concentration range = 0.8 - 2.3mM) and a constant supply of glucose is essential for proper brain functioning, hypoglycemia can compromise central nervous system (CNS) function (Gruetter et al., 1998, Pellerin, 2010). Indeed, owing to the fact that there is a linear relationship between blood and brain glucose levels (Choi et al., 2001), a severe drop in blood glucose levels - which essentially renders the brain aglycemic - can result in cognitive impairment, seizures, unconsciousness, coma and eventually neuronal cell death (Auer, 1986). In addition, researchers have found that patients that experience one or more episodes of severe hypoglycemia are at an increased risk for dementia, an impairment of cognitive function that may be associated with neuronal loss in the hippocampus (Whitmer et al., 2009).

Neuronal Vulnerability to Hypoglycemia

Along with hippocampal neurons, cortical neurons also appear to be uniquely vulnerable to injury following hypoglycemia (Auer, 2004). As both areas of the brain are rich in glutamatergic synapses, previous studies investigated the hypothesis that glutamate excitotoxicity - a process by which the excitatory amino acid glutamate overstimulates its cognate glutamate receptors and causes a calcium-dependent neuronal cell death - contributed to hypoglycemic-neuronal cell death (Olney, 1969, Choi, 1994).

Several studies utilizing the technique of microdialysis demonstrate that insulin-induced hypoglycemia results in glutamate accumulation in the rat hippocampus and striatum (Wieloch et al., 1985, Sandberg et al., 1986, Silverstein et al., 1990) and in the cerebrum of the pig (Ichord et al., 1999). Furthermore, *in vivo* deafferentation studies (Wieloch et al., 1985) along with studies showing partial protection with tetanus toxin (Monyer et al., 1992, Jackman et al., 2012) suggest that exocytotic neuronal release of glutamate contributes to hypoglycemic neuronal cell death. Additional studies, including one from our own laboratory, have determined that glutamate released from non-neuronal sources can also contribute (Kauppinen et al., 1988, Jackman et al., 2012) and these will be discussed in more detail below.

The above referenced studies, along with findings that glutamate receptor antagonists administered prior to glucose deprivation and insulin-induced hypoglycemia reduce neuronal cell death in both *in vitro* and *in vivo* models respectively, supports the role of glutamate excitotoxicity in hypoglycemic neuronal cell death (Engelsen et al., 1986, Linden et al., 1987, Monyer et al., 1989, Papagapiou and Auer, 1990, Jackman et al., 2012).

Glutamate Synthesis and Classical Release

Glutamate is an amino acid that is distributed at high concentrations throughout the CNS. Originally thought to play a purely metabolic role in the brain, it was later classified as a neurotransmitter due to its presynaptic localization, release upon physiological stimulation, and rapid termination (Krebs, 1935, Fonnum, 1984). It is now well accepted that glutamate is the main excitatory neurotransmitter in the CNS and its binding to signaling receptors underlies its role in synaptic plasticity, long-term potentiation associated with learning and memory, and cell migration and differentiation during development (Fitsanakis and Aschner, 2005). In addition to being an important and abundant neurotransmitter in the CNS, glutamate is also a metabolic precursor to the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and is a component of the antioxidant glutathione. Its synthesis is crucial for normal brain functioning, with over half of all brain synapses releasing this excitatory neurotransmitter.

Glutamate is unable to cross the blood brain barrier so it must be synthesized within cells. Glutamate can be synthesized from 2-oxoglutarate (α-ketoglutarate), a by-product of the tricarboxylic acid (TCA) cycle, through the process of transamination [for review see (Niciu et al., 2011)]. Additionally, glutamate can also be synthesized from glutamine via the glial specific enzyme glutamine synthetase (Martinez-Hernandez et al., 1977). In fact, a process of recycling glutamate known as the glutamate-glutamine cycle, which occurs mainly in astrocytes, represents an important method by which astrocytes supply neurons with the precursor for glutamate synthesis (Danbolt, 2001). Importantly, this glutamate-glutamine cycle does not interfere with neurotransmission because unlike

glutamate, glutamine is nontoxic and does not activate glutamate receptors (Danbolt, 2001).

Within neurons, glutamate is packaged into vesicles by means of a process driven by an electrochemical gradient of H^+ established by V-ATPase through the use of vesicular glutamate transporters (VGLUTs) (Takamori, 2006, Omote et al., 2011). In neurons there are three isoforms of VGLUT: VGLUT1, 2, and 3. VGLUT1 and 2 are expressed in glutamatergic neurons with VGLUT1 predominantly expressed in the neocortex, striatum, piriform cortex, hippocampus, thalamus and cerebellum and VGLUT2 predominantly expressed in the nucleus accumbens and hypothalamus (Liguz-Lecznar and Skangiel-Kramska, 2007). VGLUT3 has been shown to be expressed in non-glutamatergic neurons and its exact function has yet to be determined [for review see (Niciu et al., 2011)].

Once glutamate has been packaged in the neuronal cell, it can be released upon electrical stimulation. Following the arrival of an action potential at the nerve terminal, voltage activated calcium channels (VACCs) open and allow for an influx of Ca^{2+} ions into the nerve terminal. This influx of Ca^{2+} allows for the assembly of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) complex, which facilitates vesicle fusion with the plasma membrane and exocytosis of vesicular content (Freberg, 2006, Pang and Sudhof, 2010).

Glutamate Signaling

There are two main groups of glutamate receptors in the CNS; ionotropic and metabotropic. Glutamate acts on three types of ionotropic receptors: kainite (KA), DL-a-

amino-2-hydroxy-5-methylisoxasole-4-propionate (AMPA), and N-methyl-D-aspartate (NMDA), which have been distinguished on the basis of their pharmacological and electrophysiological properties as well as by molecular cloning (Hollmann and Heinemann, 1994). All three ionotropic glutamate receptors (iGluRs) are glutamate-gated cation channels. Of these, NMDA receptors differ from the others as they are not purely ligand gated but also require depolarization for activation. This is due to the fact that NMDA receptors contain a magnesium (Mg^{2+}) ion blocking current entry through the ion channel pore that must be removed by cell depolarization in order for the channel to open. The activation of AMPA and kainate receptors through glutamate binding allows for the influx of sodium, neuronal depolarization and removal of the Mg^{2+} block (Mayer et al., 1984).

Metabotropic glutamate receptors (mGluRs) differ from iGluRs in that they are not glutamate-gated ion channels but rather, transmembrane G-protein coupled receptors. The mGluRs (mGluR1-8) are subclassified into three groups based on their structure, pharmacology and transduction mechanisms: group I (mGlurR1 and mGlur5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8). Group I mGluRs are coupled to phospholipase C (PLC) and associated with the release of Ca^{2+} from intracellular stores while groups II and III are negatively coupled to adenylate cyclase, inhibiting voltage-sensitive Ca^{2+} channels and activating K^{+} channels (Pin and Acher, 2002).

The location of the GluRs (i.e., presynaptic vs. postsynaptic) in the CNS contributes to the specific roles that they play in synaptic modulation. For example, KA receptors located on presynaptic hippocampal neurons have been shown to act as

autoreceptors that facilitate and enhance short term synaptic plasticity as well as heteroreceptors that enhance GABA release from local interneurons, resulting in an overall inhibitory effect (Pinheiro and Mulle, 2008). Endogenous glutamate binding to presynaptic neuronal NMDA receptors increases the frequency of excitatory postsynaptic potentials (EPSPs) in the cerebellar cortex and AMPA receptors activated presynaptically result in neuronal depolarization. In contrast, activation of presynaptic mGluRs typically decrease excitatory glutamatergic neurotransmission (Pinheiro and Mulle, 2008).

Glutamate binding to postsynaptic iGluRs has an excitatory effect while activation of postsynaptic mGluRs have a multitude of functions. In general, activation of group I mGluRs results in depolarization and excitation of the postsynaptic neuron while groups II and III (with the exception of mGluR6 located presynaptically) less commonly activate postsynaptically (Niswender and Conn, 2010).

Interestingly, astrocytes also express glutamate receptors and possess functional iGluRs as well as mGluRs [for review see (Parpura and Verkhratsky, 2012)]. Multiple *in situ* studies have shown that functional AMPA and NMDA receptors are expressed on astrocytes in the hippocampus (Porter and McCarthy, 1995, Seifert and Steinhauser, 1995) and cortex (Conti et al., 1999, Lalo et al., 2006) as well as the Bergmann glia of the cerebellum (Muller et al., 1992, Luque and Richards, 1995). In addition to mGluRs being expressed in astrocytes in various brain tissues (Gallo and Russell, 1995), it has also been shown that Group 1 mGluRs are expressed in the hippocampus (Cai et al., 2000). The fact that a variety of GluRs are expressed in both astrocytes and neurons indicates that bidirectional signaling between astrocytes and neurons as well as between pre- and

postsynaptic neurons plays an important role in glutamatergic signaling throughout the CNS.

Glutamate Removal

Neurotransmitter signaling, in general, can be terminated by a variety of means including diffusion, enzymatic inactivation and uptake into neurons and/or astrocytes (Bauer, 1985). In the case of glutamate, no enzymes have been found to metabolize glutamate in the synaptic cleft. Instead, glutamate in the CNS is removed rapidly from the extracellular space actively across its concentration gradient by excitatory amino acid transporters (EAATs). EAATs are responsible for keeping extracellular levels of glutamate below excitotoxic levels; more specifically, EAATs in astrocytes are predominantly responsible for the removal of glutamate from the extracellular space [for review see (Anderson and Swanson, 2000)].

During normal physiological conditions, the concentration of intracellular glutamate is usually 1-10 mM while extracellular glutamate concentrations in the CNS are typically 2 µM or less (Benveniste et al., 1984, Erecinska and Silver, 1990). Glutamate is taken up into the cell using the co-transport of three Na^+ ions and one H^+ ion into the cell and the counter transport of one K^+ ion outward (Zerangue and Kavanaugh, 1996b). Neurons express the sodium- and potassium-coupled glutamate transporters EAAT3 (EAAC1), EAAT4, and EAAT5. Neuronal glutamate transporters also contribute to glutamate removal in the CNS, with EAAT3 located in neuronal somata and dendrites, EAAT4 in cerebellar purkinje cells, and EAAT5 in retinal photoreceptors and bipolar

cells (Attwell, 2000). However, glutamate clearance in the CNS occurs primarily via astrocyte glutamate transporters (Anderson and Swanson, 2000).

The astrocyte EAATs that are responsible for maintaining normal extracellular glutamate concentrations are EAAT1 and EAAT2, also known as GLAST and GLT-1 respectively (Danbolt, 2001, Beart and O'Shea, 2007). These two transporters differ in their distribution in the CNS, with GLAST being most abundantly expressed in Bergmann glia and GLT-1 being most abundant in the forebrain regions (Anderson and Swanson, 2000). Both GLT-1 and GLAST are enriched in astrocytic processes and the antisense knockdown of either transporter results in excitotoxicity and increased susceptibility to seizures and neuronal injury (Rothstein et al., 1996, Nag, 2011). Excitotoxicity has been shown to be an important mechanism of neuronal death in trauma, ischemia, and hypoglycemia, further stressing the necessity of GLT-1 and GLAST (Choi, 1988).

In addition to the high-affinity EAATs, another method of Na^+-dependent high-affinity glutamate uptake is achieved through neutral amino acid transporters ASCT1 and ASCT2, which exchange electroneutral amino acids such as glutamate, alanine, serine, cysteine, and threonine and exhibit a substrate-modulated chloride conductance (Arriza et al., 1993, Zerangue and Kavanaugh, 1996a).

Potentially Pathological Non-classical Non-Neuronal Release of Glutamate

While the most commonly discussed method of glutamate release is by means of action potential-stimulated exocytotic release from presynaptic neurons (discussed above), there are a variety of other release mechanisms, occurring most prominently from

astrocytes, that can contribute to an increase in extracellular glutamate levels, particularly under pathological conditions. Glutamate has been described to be released via a calcium-dependent mechanism akin to exocytosis in neurons, via reversal of glutamate transporters designed for uptake, via swelling-activated anion channels (Kimelberg et al., 1990), via unopposed hemichannels, via the activation of the $P2X_7$ receptor, and via the cystine-glutamate exchanger, system x_c^- [for review see (Verkhratsky and Parpura, 2010)].

Astrocytes are incapable of communication through electrical signals, although they are able to convert external inputs to intracellular calcium signaling (Santello and Volterra, 2009). Ligands binding to G protein-coupled receptors on the membrane of astrocytes result in the release of Ca^{2+} from internal stores. Similar to exocytosis in neurons, this elevated intracellular Ca^{2+} has been demonstrated to support exocytotic release of glutamate from the astrocyte. Instead of classical synaptic vesicles, glutamate-containing synaptic-like microvesicles (SLMVs) are fused to the astrocyte membrane and glutamate is released into the extracellular space (Bezzi et al., 2004).

Reversal of glutamate transporters normally responsible for removal of extracelluar glutamate (see above) can also contribute to release of glutamate into the extracellular space. In particular, the reversal of EAAT1 and EAAT2 in astrocytes has been demonstrated to occur and to contribute to extracellular glutamate levels under pathological conditions such as ischemia, metabolic blockade (i.e., situations mimicking hypoglycemia), or perturbed ionic conditions (Szatkowski et al., 1990, Longuemare and Swanson, 1995, Zeevalk et al., 1998, Seki et al., 1999, Rossi et al., 2000).

Under hypo-osmotic conditions, release of glutamate from volume regulated anion channels (VRACS) induced by swelling also has been demonstrated (Kimelberg et al., 1990, Kimelberg and Mongin, 1998). VRACs are located in plasma membranes and allow inorganic and small organic anions including glutamate to cross the plasma membrane into the extracellular space (Mongin and Orlov, 2001). Specifically, the volume-sensitive outwardly rectifying (VSOR) chloride channels and the maxi-anion channels have been implicated in astrocytes as contributing to an efflux of glutamate following astrocytic swelling (Liu et al., 2006). This is thought to be especially important under ischemic conditions (Malarkey and Parpura, 2008).

Some, but not all, astrocytes are connected to each other through gap junctions that allow molecules to pass through between cells. Gap junctions are composed of opposed hemichannels consisting of hexamers of connexins or pannexins. These hemichannels are capable of passing large molecules and under conditions of low extracellular divalent cations have been shown to release glutamate (Ye et al., 2003). An unopposed connexin can open to the extracellular environment thereby becoming a conduit for glutamate release. Studies have shown that connexin hemichannels in astrocytes, predominantly connexin 43, are capable of releasing glutamate (Ye et al., 2003). Furthermore, pannexin has been found to be insensitive to extracellular Ca^{2+} and to be opened in response to elevations in intracellular Ca^{2+} levels (Bruzzone et al., 2003). Lastly, studies in neurons have shown that hemichannels release glutamate under ischemic conditions (Thompson et al., 2006). Therefore, hemichannels can be considered another source of glutamate release in the CNS.

In addition, it should also be noted that research on heterocarriers suggests that stimulation of one type of transporter may release glutamate via a different carrier and this could be another proposed method of glutamate release (Bonanno et al., 1993). Studies have also suggested a role for ATP-activated $P2X_7$ receptors in glutamate release in astrocytes (Duan et al., 2003).

Finally, system x_c^- , a transmembrane protein that exchanges intracellular glutamate for extracellular cystine has been shown to contribute to neuronal injury in neurodegenerative diseases of the CNS including Alzheimer's disease (Barger and Basile, 2001) and Parkinson's disease (Massie et al., 2011), in white matter disease such as multiple sclerosis (Domercq et al., 2007), and in the neurotoxicity associated with glial tumors (Chung et al., 2005). Our laboratory recently demonstrated its neuropathological potential in *in vitro* models of hypoxia and hypoglycemia (Fogal et al., 2007, Jackman et al., 2010, Jackman et al., 2012).

System x_c^-

The knowledge that hypoglycemic neuronal injury is a result of excitotoxicity and that there are many sources of glutamate in the CNS means that glutamate from a variety of sources could contribute to hypoglycemic neuronal cell death. Our laboratory has focused on the potential role of astrocyte system x_c^-, an anionic antiporter shown to release glutamate in the CNS, in hypoglycemic neuronal cell death. System x_c^- is a sodium independent heterodimeric amino acid transporter consisting of a specific light chain, xCT, and a heavy chain, 4F2hc. The heavy chain 4F2hc is a promiscuous transmembrane protein that is a component of other amino acid transporters and

responsible for the localization of system x_c^- to the membrane of cells. The light chain xCT confers substrate specificity, with the overexpression of the 12 transmembrane domain xCT alone resulting in an increase in system x_c^- activity (Shih et al., 2006). System x_c^- is a cystine/glutamate antiporter that exchanges intracellular glutamate for extracellular cystine in a 1:1 ratio. System x_c^- has been shown to be constitutively expressed throughout the brain, thymus and spleen in mice (Sato et al., 2002). With respect to the nervous system, system x_c^- has been described in astrocytes (Cho and Bannai, 1990, Jackman et al., 2010), cortical neurons (Murphy et al., 1990, Jackman et al., 2010) and microglia (Piani and Fontana, 1994, Jackman et al., 2010) of the CNS. The role for system x_c^- in the synthesis of the antioxidant glutathione (GSH) (Bannai, 1986) is well documented (Dringen and Hirrlinger, 2003) and its role in the maintenance of the ambient extracellular level of glutamate has been demonstrated (Augustin et al., 2007, Featherstone and Shippy, 2008, De Bundel et al., 2011, Massie et al., 2011).

Regulation of System x_c^-

During times of high oxidative stress, the demand for GSH is increased and the stress-inducible transcription factor Nrf2 is able to upregulate xCT (Shih et al., 2003). Numerous studies have demonstrated that xCT can be regulated by stimuli such as ROS, electrophilic agents, heavy metals, and cytokines at the transcriptional level (Bannai, 1984, Ishii et al., 2000, Sasaki et al., 2002, Jackman et al., 2010). This upregulation of xCT leads to an increase in system x_c^- activity and consequently a more rapid efflux of glutamate from astrocytes as a byproduct of their attempt to utilize cystine uptake to produce GSH (Piani and Fontana, 1994), a process designed to be beneficial but under

certain conditions (as described above) becomes harmful in the CNS. To this end, animal models have been designed to look at the role of system x_c^- in disease.

Animal Models for the Study of System x_c^- Activity

In 2005, Chintala et al. characterized mice harboring a spontaneous deletion in the region encoding for xCT, the *Slc7a11* gene. These subtle gray (*sut*) mutants, characterized by a reduced yellow pigmentation, lack functional xCT and exhibit very little cystine uptake. Furthermore, cultures from these mice require the addition of the reducing agent β-mercaptoethanol (β-ME), supporting evidence of a reduction in GSH production in these mice (Chintala et al., 2005). Also in 2005, Sato and colleagues developed a genetically engineered xCT knockout mouse in which the START codon of the *Slc7a11* gene was deleted. These mice lack detectable xCT transcript and do not show system x_c^- mediated L-cystine uptake (Sato et al., 2005). These mice are valuable for the study of the role of system x_c^- in health and disease and our laboratory has more specifically utilized *sut* mice in previous investigations to dissect out the role of astrocytes system x_c^- in hypoxic and aglycemic neuronal injury (Jackman et al., 2010, Jackman et al., 2012).

System x_c^- and Hypoglycemic Excitotoxic Neuronal Injury

As described above, there are a plethora of pathways, both neuronal and non-neuronal, which can support potentially pathologic glutamate release. Previous studies in our laboratory utilizing both a pharmacological and genetic approach demonstrated that as expected aglycemic neuronal cell death is mediated by NMDA receptor

overstimulation (Jackman et al., 2012). By utilizing a cortical co-culture system to assess the cellular and molecular pathways by which glutamate was released, we described a prominent role for astrocyte system x_c^- (Jackman et al., 2012). Specifically, we found that removal of amino acids during the deprivation period prevents - whereas addition of L-cystine restores - glucose deprivation-induced neuronal death, implicating system x_c^-. Additionally, drugs known to inhibit system x_c^- ameliorated glucose deprivation-induced neuronal death. While these studies indicated that system x_c^- was the source of excitotoxic glutamate during glucose deprivation, it was the use of chimeric cultures derived from the *sut* mice mentioned above that showed which cell type was responsible for aglycemic neuronal cell death. In these studies, a significant decrease in aglycemic neuronal cell death was seen when astrocytes derived from *sut* mice were cultured with wild type neurons when compared to mixed cortical cultures of wild type astrocytes and neurons, implicating astrocyte system x_c^- as the source of excitotoxic glutamate during aglycemic conditions (Jackman et al., 2012).

However, the precise mechanism by which system x_c^- activity links to glutamate-mediated injury has yet to be determined. Thus, the overall purpose of this thesis was to investigate whether changes in system x_c^- expression in our astrocyte and mixed cortical cell cultures and/or alterations in glutamate handling in a mixed cortical culture model following glucose deprivation occur(s) and contributes to aglycemic neuronal cell death.

Materials and Methods:

Cell culture

Media stock contained the following: glutamine-free Eagle's minimum essential media, 25.7 mM glucose, 28.2 mM sodium bicarbonate and was supplemented with 2.0 mM L-glutamine prior to use in experiments. Astrocyte plating media consisted of media stock supplemented with 10% fetal bovine serum (FBS), 10% bovine calf serum (BCS), 10 ng/ml epidermal growth factor, 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.0 mM L-glutamine. Neuronal plating media consisted of media stock supplemented with 5% BCS, 5% bovine growth serum (BGS), 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.0 mM L-glutamine. Where indicated, cells were maintained in a maintenance media composed of media stock supplemented with 10% BCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.0 mM L-glutamine.

Primary astrocytes were cultured from cortices of postnatal CD-1 pups (1-3 days; Charles River) as described in detail previously (Uliasz et al., 2012). In brief, following an asceptic dissection, cerebral cortices were placed in 0.025% trypsin diluted in Hank's balanced salt solution (Mediatek) and incubated for 15 min at 37°C. Dissociated cells were pelleted via centrifugation (3 min, 760 *g*), medium decanted and cells resuspended in astrocyte plating medium and plated at the equivalent of 2 hemispheres per 10 ml in 15 mm 24 well plates (Falcon, Primaria). Upon confluence, monolayers of astrocytes were treated with 8 µM β-D-cytosine arabinofuranoside (AraC) for 6-7 days to prevent microglial cell growth. To remove any residual microglia, monolayers were incubated with 50 mM of leucine methyl ester for 30-35 min one day prior to experimentation.

Primary near-pure neuronal cultures were derived from the cortices of embryonic day 15 CD1 mice, essentially as described above for astrocytes except that they were cultured at a density of 1 million cells per ml and plated onto polyethyleneimine coated plates in a neuronal plating media for just four hours. Thereafter, media was exchanged and cells placed in neurobasal media supplemented with 1X B27, 2.0 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. After two days, neurons were treated with 1 µM of AraC once for 2 days to prevent glial cell growth. Media was partially replenished two days later by exchanging 250 µl of media with fresh neurobasal media containing supplements. Pure neuronal cultures were used at 6 days *in vitro*.

Mixed cortical cell cultures consisting primarily of neurons and astrocytes were prepared by plating dissociated cortical cells (4.5 hemispheres per 10 ml) from embryonic day 15 CD1 mouse fetuses on a confluent layer of astrocytes in neuronal plating media. The medium was partially replaced after 5 and 9 day *in vitro* and treated with 8 µM of AraC at 7 days *in vitro.* Two days prior to experimentation mixed cortical cell cultures were placed into media stock. Experiments were performed at 14 days *in vitro*. All cultures were kept at 37°C in a humidified 6% CO_2-containing incubator.

Glucose deprivation

To remove glucose from the mixed cortical cells, 250 µl of media was removed from the cultures before the addition of 750 µl BSS_0, so as to not expose neurons to the air. After a total of 8 X 750 µl washes, cultures were left in BSS_0 (400 µl). To deprive astrocytes of glucose, medium was completely aspirated, as a brief exposure to air does not harm astrocytes, and cultures were washed 2 X 400 µl with BSS_0 and then left in BSS_0 (400 µl) for the duration of the experiments. In all experiments, parallel cultures

were supplemented with 10 mM glucose to serve as controls. Inhibitors were given at the initiation of glucose deprivation except in the case of time course experiments whereby drugs were added as indicated in the figure legend.

Measurement of cell death

Cell death was determined via quantification of the amount of lactate dehydrogenase (LDH) released into the cell culture medium by measuring the pyruvate-dependent NADH oxidation spectrophotometrically at 340 nm (Uliasz and Hewett, 2000). Neuronal cell death was expressed as a percentage of total neuronal LDH activity obtained by exposing parallel cultures to 200μM of NMDA for 20-24h. Cultured astrocytes do not express NMDA receptors (Backus et al., 1989, Chan et al., 1990) and have previously been shown to survive up to 8 h of glucose deprivation (Jackman et al., 2012), therefore these LDH measurements specifically reflect neuronal cell death only.

qPCR

Total RNA was extracted from samples (four wells from 24-well plates pooled) using TRIzol reagent (Invitrogen) and suspended in 20 μl of RNase-free water. RNA was quantified spectrophotometrically at 260 nm via NanoDrop® and first-strand cDNA synthesized from 1 μg RNA using M-MLV transcriptase, oligo (d)T primers, dNTPs, DTT, and MMLV buffer (Invitrogen) as described in detail (Hewett et al., 1999, Jackman et al., 2012). qPCR was performed using Taqman Gene Expression Assays' mouse-specific primer pairs (xCT: Mm00442530_m1, β-actin: Mm01205647_g1) per manufacturer's instructions and relative quantification performed using the comparative cycle threshold method ($\Delta\Delta C_T$) where C_T values from experimental samples were normalized to β-actin C_T values from the same sample, and then compared to a calibrator

sample C_T value (cultures kept in BSS_{10}) to determine the relative fold increase in mRNA.

Toxicity bioassay

In order to determine whether glucose deprived cells still retained the capacity for glutamate uptake, we utilized a toxicity bioassay that our laboratory previously determined to be a sensitive measure of changes in EAAT activity in astrocytes (Sen et al., 2011). Seventy-five µM glutamate was spiked into pure astrocyte cultures containing glucose or into those that were glucose-deprived for 6 h and left for a total of 60, 75, 90 or 105 min. Media was then removed and like conditions were pooled. Prior to placing on pure neuronal cultures to determine the residual toxicity of the astrocyte exposure medium, glucose (10mM) was added to the media removed from glucose-deprived cultures. Media was partially exchanged by removing 250 µL of neuronal culture media and adding 250µL of pooled experimental media to pure neuronal cultures at 6 days *in vitro*. Neuronal cell death was assessed after 6 h and expressed as a percentage of total neuronal cell death by exposing parallel cultures to 200 µM glutamate for 20-24 h (100% neuronal cell death).

Immunoblotting

Cells from mixed and astrocyte cultures (six wells from a 24-well plate pooled) were harvested by gentle scraping followed by centrifugation at 600 *g* for 6 min. The supernatant was then discarded and the pellet was washed with 3 ml of ice cold PBS before centrifugation (600 *g*; 3 min) and storage at -80°C. Upon thawing, 100 µl of standard RIPA Buffer containing 50 mM Tris, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P40, 150 mM NaCl, 5 mM iodacetamide, 5 mM, EDTA, 5 mM EGTA, and 1X

complete protease inhibitor cocktail (Roche Diagnositcs) was added to the tubes, which were then incubated at 4°C for 30 min. Cellular debris was removed via centrifugation (12,000 *g*; 20 min; 4°C). Protein concentration of the supernatants was determined (BCA assay; Pierce) and 30 µg (mixed culture) or fifteen µg (astrocyte culture) were separated by SDS-PAGE (11% polyacrylamide gel) under reducing conditions and then proteins electrophoretically transferred to a PVDF membrane (Millipore). Membranes were blocked in Odyssey Blocking Buffer (LI-COR) for 1 h before incubation with primary antibodies overnight at 4°C (xCT; 1 µg/ml, rabbit polyclonal, Novus Biologicals; β-actin; 0.3 µg/ml, mouse monoclonal; Sigma). Membranes were washed 4 X 10 min with PBST (1X PBS with 0.1% Tween 20) before incubation with fluorescent-conjugated secondary antibodies (goat anti-rabbit IRDye 680LT and goat anti-mouse IRDye 800CW, respectively) for 1 h at room temperature. Membranes were then washed 4 X 10 min with PBST, and 1 X 10 min with PBS to remove residual tween before protein was detected using the LI-COR Odyssey system.

NMDA and glutamate excitotoxicity assays

In order to determine whether there was a difference in neuronal sensitivity to NDMA receptor agonists following glucose deprivation, our mixed cortical cultures were either glucose deprived or kept in glucose-containing media before being exposed to NMDA and glutamate. Mixed cortical cultures were deprived of glucose for two hours and fifteen minutes before being washed (2 X 750 µl HBSS) and exposed to increasing concentrations of either NMDA or glutamate in HBSS for 10 min. In the case of the time course experiments, mixed cortical cultures were glucose-deprived for various amounts of time as indicated in Figure 6 before being washed and exposed to either 25 µM

NMDA or 100 µM glutamate for 10 min. All cultures were then washed with HBSS, put into medium stock and incubated at 37°C, 6% CO_2 overnight before assessment of neuronal cell death via the LDH assay.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism Version 6.0 as described. Percentage data were transformed (arcsin square root) before analysis because it is non-normally distributed. Values of zero or less were set at $1 \text{ X } 10^{-20}$ before transformation. In the case of qPCR, statistics were performed on the geometric means of $2^{-\Delta\Delta CT}$ values. Significance was assessed at $P < 0.05$.

Results:

Consistent with prior literature from our own laboratory as well as that of others, we demonstrate that aglycemic neuronal cell death occurs in a time dependent manner (Figure 1A) and is a result of glutamate excitotoxicity (Figure 1B) (Monyer and Choi, 1988, Monyer et al., 1989, Jackman et al., 2012). Specifically, neurons in mixed cortical cell culture are unaffected by up to 3 h of glucose deprivation, but significant death occurs following deprivation times of 6 and 8 h (Figure 1A). This injury is completely prevented by the addition of the non-competitive NMDA receptor antagonist, MK-801 (Figure 1B). The source of glutamate appears to be the cystine/glutamate antiporter, system x_c^-, as aglycemic neuronal cell death is prevented when amino acids (Figure 1C) - more specifically cystine (Jackman et al., 2012) - are removed from the glucose free medium or when a system x_c^-antagonist, (LY367385) is added (Figure 1C; Jackman et al., 2012).

Although, both NMDA receptor and system x_c^- antagonism prevent neuronal cell death when given at the start of glucose deprivation, their protective therapeutic window of opportunity is not known. Toward this end, mixed cortical cultures were deprived of glucose for a total of 8 h and either MK-801 (Figure 2A) or LY367385 (Figure 2B) was added to parallel cultures at one hour intervals post-experimental onset (Figure 2). When compared to 8 h, both inhibitors were fully able to block aglycemia-induced neuronal cell death when added up to 2 h post-deprivation (Figure 2A,B). Remarkably, neurons showed partial, but significant, protection even when addition of the inhibitors was delayed for up to six h (Figure 2A,B), an effect lost when administration was delayed to 7 h (Figure 2A,B).

Despite pinpointing the molecular source of excitotoxic glutamate, the mechanism by which injury is facilitated is not well understood. Numerous studies have demonstrated that the specific light chain of system x_c^-, xCT, can be regulated by stimuli such as ROS, electrophilic agents, heavy metals, and cytokines at the transcriptional level (Bannai, 1984, Ishii et al., 2000, Sasaki et al., 2002, Jackman et al., 2010). Hence, whether xCT mRNA levels were altered following aglycemia in pure astrocyte cultures or in mixed cortical cultures containing both astrocytes and neurons was next assessed. Real time PCR analysis of xCT levels that follow one to 8 h of glucose deprivation revealed a significant increase in xCT mRNA at the 8 h time point only (Figure 3A, B).

This change notwithstanding, western blot analysis, of either astrocyte or mixed cultures, demonstrate no change in xCT protein expression over the identical time course (Figure 4 A,B). In keeping with this latter finding, cycloheximide (1 μg/mL), when given at the onset of glucose deprivation, failed to prevent aglycemic neuronal cell death (Figure 5A), indicating that this injury was independent of protein synthesis. Additionally, death was not prevented by actinomycin D (10 μg/ml), demonstrating no requirement for transcription (Figure 5B). Since maintenance of extracellular glutamate concentrations within a narrow physiological range is important to avoid excitotoxicity and aglycemic alterations in system x_c^- expression do not appear to underlie the neurotoxicity associated with glucose-deprivation, investigations into whether alterations in glutamate uptake existed ensued.

To determine whether EAAT activity was impaired following glucose deprivation, the glutamate buffering capacity of astrocytes was assessed (Sen et al., 2011). For these studies, glutamate (final concentration = 75 μM) was added to control

astrocytes or astrocytes deprived of glucose (6 h) and at various times post-addition the medium was collected, and then transferred to pure neuronal cultures for six h, to assess for neuronal cell death. As expected, the longer the glutamate-containing media was left on control astrocyte cultures the less neuronal cell death occured in the pure neuronal cultures (Figure 6). Interestingly, glucose-deprived cultures appear to have the same glutamate buffering capacity as control astrocytes as glutamate-containing media removed from glucose-deprived (6 h) astrocytes also demonstrated a time-dependent decrease in neuronal cell death and, importantly, was equally toxic to pure neuronal cultures at every time point assessed (Figure 6).

Since, our results indicated no change in system x_c^- expression or glutamate uptake under aglycemic conditions, it was hypothesized that glucose-deprived neurons may simply be more sensitive to the injury causing effects of glutamate receptor stimulation. Toward this end, we first used the agonist NMDA, which cannot be taken up or metabolized by cells, and found that equal concentrations of NMDA killed significantly more glucose-deprived neurons in mixed cortical cultures than control cells after a ten minute exposure (Figure 7A). Interestingly, this difference in sensitivity to NMDA was significant over multiple time points (Figure 7B). Next we investigated the effects of glutamate in mixed cortical cultures in a similar manner and found that equal concentrations of glutamate killed significantly more neurons in glucose-deprived cultures (Figure 8A) as well as across various time points (Figure 8B). From these results we concluded that neurons in the mixed cortical cultures deprived of glucose were more sensitive to the toxic effects of glutamate receptor stimulation than the neurons in mixed cultures with glucose-containing media

Discussion:

Hypoglycemia is a serious medical threat to people with diabetes mellitus and is a limiting factor in the treatment and regulation of blood glucose levels. Despite efforts of medical physicians and patients using glucose-lowering drugs to balance the importance of glycemic control with the avoidance of hypoglycemia, tight glucose regulation can lower plasma glucose concentrations such that the patient experiences brain dysfunction (Cryer, 2001). Interestingly, the underlying cause of the severe neurological complications that follow a dangerous drop in blood glucose levels is not directly due to energy failure but appears to be a result of neuronal cell death that ensues secondary to excitotoxicity, which is most commonly due to over-excitation of neuronal glutamate receptors (Monyer et al., 1989, Papagapiou and Auer, 1990, Choi, 1994). While multiple sources of glutamate may contribute [i.e., non-neuronal and neuronal and vesicular or non-vesicular (Danbolt, 2001)], evidence from our laboratory implicated glutamate efflux from astrocytes via system x_c^- as a contributory factor to glucose-deprivation induced cell death (Jackman et al., 2012). The precise mechanism by which system x_c- activity linked to glutamate-mediated neuronal injury was not determined.

Hence, the overall purpose of this thesis was to investigate whether changes in system x_c^- expression in astrocyte and mixed cortical cell cultures and/or alterations in glutamate handling in a mixed cortical culture system following glucose deprivation occur(s) and contributes to aglycemic neuronal cell death. The major findings of this work are as follows: 1) alterations in xCT expression (mRNA and protein) and function (inferred) do not contribute to aglycemic neuronal injury; 2) alterations in classical glutamate re-uptake transporter function do not appear to contribute to aglycemic

neuronal cell death; 3) glucose-deprived neurons are more sensitive to the toxic effects of either glutamate or NMDA than control cultures containing glucose; and 4) utilizing inhibitors of glutamate receptors or system x_c^- , a possible extended therapeutic window of opportunity for protection was established.

In order to investigate whether changes in system x_c^- expression occur following glucose deprivation, we first compared xCT mRNA levels between glucose-deprived and glucose-containing primary astrocytes alone or in mixed cortical cultures containing both astrocytes and neurons, for it is well known that several stimuli can regulate xCT transcription *in vitro*. These stimuli can be as diverse as ROS and amino acid starvation each acting via different promoter elements namely the electrophile response element (EpRE) and the amino acid response element (AARE), respectively (Sasaki et al., 2002, Lewerenz and Maher, 2009). Whether glucose deprivation could similarly alter transcription has never been established.

Using quantitative PCR, this study demonstrated that xCT mRNA does indeed increase in both culture systems following glucose deprivation. However, this only occurs after 8 h, which is a time by which significant, and in most cases, maximal neuronal cell death in the mixed cortical cell culture system has already occurred. Thus, it seems unlikely that changes in system x_c^- expression, and by inference function, could be contributing to the aglycemic neuronal cell death. In support of this conclusion, neither actinomycin D nor cycloheximide provided any protection from aglycemia-mediated neuronal cell death, demonstrating that neither transcription nor translation was required. In further support, no change in xCT protein expression for up to 8 h of glucose deprivation was demonstrated by western blot analysis. We fully acknowledge that

changes in functional protein at the membrane level could occur independent of protein synthesis. For example, studies show that there is a possibility for the activity of system x_c^- to be regulated via phosphorylation of putative PKA sites in human xCT (Baker et al., 2002). Furthermore, changes in protein trafficking could lead to changes in xCT protein insertion in the membrane that would not be detected through the measurement of total xCT protein within the cell (this study). However, using a radiolabeled cystine uptake assay, a previous graduate student in the laboratory directly demonstrated that no change in system x_c^- activity occurs following glucose deprivation (unpublished observations), thus supporting our contention that changes in system x_c^- do not underlie its neurotoxicity under the condition of glucose deprivation.

Likewise, utilizing a toxicity bioassay developed by us and validated for its ability to accurately reflect glutamate uptake into astrocytes (Sen et al., 2011), we saw no change in the ability of glucose-deprived astrocytes alone or astrocytes contained in mixed culture to remove glutamate from the extracellular environment, seemingly ruling out loss of uptake and hence a build-up of extracellular glutamate as a contributory factor. While this might initially seem surprising, others have shown that there were no changes in astrocytic glutamate uptake in astrocytes for up to two hours of glucose deprivation (Bakken et al., 1998) and only a 20% loss after 24 h (Swanson and Benington, 1996). Indeed, astrocytes have been demonstrated to be more resistant (Monyer and Choi, 1988, Monyer et al., 1989, Goldberg and Choi, 1993, Lyons and Kettenmann, 1998) to the ATP-depleting effects of glucose starvation by virtue of their ability to utilize glycogen stores (Cataldo and Broadwell, 1986, Swanson et al., 1990) that can be metabolized to meet their metabolic needs (Swanson et al., 1990, Erecinska

and Silver, 1994, Dienel and Cruz, 2006, Walls et al., 2009). Additionally, the ability of astrocytes to convert glutamate to pyruvate to support TCA cycling has been demonstrated to occur when glucose levels are low (Bakken et al., 1998).

Thus, loss of astrocyte glutamate uptake may not fully explain the enhancement of extracellular glutamate levels in rat striatum and the hippocampus and pig cerebrum (Wieloch, 1985, Sandberg et al., 1986, Silverstein et al., 1990) (Ichord et al., 1999) measured *in vivo* via microdialysis probe following insulin-induced hypoglycemia in rat. An important caveat to microdialysis studies to bear in mind is that probe insertion itself can result in tissue damage altering the accuracy of the measurements obtained (Westergren et al., 1995, Clapp-Lilly et al., 1999); however, we similarly acknowledge the limitations of interpretation using cell culture models.

Finally, it is important to point out that neither enhanced release of glutamate nor its diminished uptake may be needed to facilitate aglycemic neuronal cell death, as Novelli and colleagues demonstrated nearly 20 years ago that glutamate concentrations needed to kill energy-deprived cerebellar neurons are far less than those required to kill healthy neurons (Novelli et al., 1988, Henneberry et al., 1989). Thus, we hypothesized that aglycemic cortical neuronal cell death may not require a change in glutamate handling but that normally innocuous levels of extracellular glutamate may become cytotoxic under conditions of glucose deprivation. Indeed, we found that glucose deprived cultures showed a two-three fold enhancement in neuronal cell death when exposed to 25 μM NMDA at nearly every time point of glucose-deprivation analyzed (1.5-4.5 h) when compared to their glucose containing controls. Similar results were seen when100 μM glutamate was utilized. These data may best be explained by the the weak

alternative excitotoxic hypothesis that Ablin and Greenamyre proposed in 1992 by which low levels of excitotoxic amino acids would be able to cause excitotoxicity due to changes in neuronal cell metabolism that leave them more susceptible (Albin and Greenamyre, 1992).

In conclusion, this current study confirms that system x_c^- is responsible for aglycemic neuronal cell death and proposes that this excitotoxic neuronal cell death is not a result of changes in system x_c^- regulation or the impairment of glutamate removal. Instead our studies show that glucose deprived neurons appear to be more susceptible to glutamate insult and hypothesize that basal levels of glutamate released from system x_c^- are responsible for aglycemic neuronal cell death.

Future Directions:

It is well known that several cell and molecular sources of glutamate in the CNS exist. While our laboratory has demonstrated previously that astrocyte system x_c^- activity is mainly responsible for the initiation of aglycemic neuronal cell death (Jackman et al., 2012), these data were not confirmed herein. Hence, chimeric cultures derived from combinations of *sut/sut* (i.e. cells lacking xCT) and +/+ cells will be produced in order to confirm the role of astrocytes and assess any contribution of neuronal system x_c^-.

Although we concluded and are confident that no alterations in system x_c^- expression occurs in our cell culture system, this inference was based on a lack of change in total cellular xCT levels. Specifically, investigating the membrane xCT protein levels using biotinylation and/or membrane fractionation methods will provide more information concerning the possible regulation of xCT insertion that could occur independent of transcription or translation.

Concerning our conclusions regarding unaltered glutamate uptake into astrocyte or mixed cultures, future studies aimed at measuring extracellular glutamate concentrations following glucose deprivation would provide direct evidence for whether or not glutamate accumulates following aglycemia.

Based on our results and other literature it will be beneficial for future experiments to investigate the underlying mechanism responsible for the enhanced susceptibility in glucose deprived cultures to glutamate. One hypothesis is that the decrease in glucose as a valuable neuronal energy source leads to a decrease in ATP levels that alters the ability of neurons to extrude calcium via its calcium-ATPase resulting in calcium accumulation and neuronal cell death.

Finally, while our findings provide novel information about the effects of glutamate and NMDA in a physiologically relevant *in vitro* mixed culture model of aglycemia, future studies utilizing *in vivo* methods would provide a better understanding of the therapeutic relevance of the inhibition of system x_c^- during severe hypoglycemia in diabetic patients. An insulin-induced hypoglycemic model in animals lacking xCT, specifically in astrocytes alone, would be optimal in order to determine the protective effects of eliminating astrocyte system x_c^- activity during hypoglycemia. Such a mouse is being developed by our laboratory at the present time.

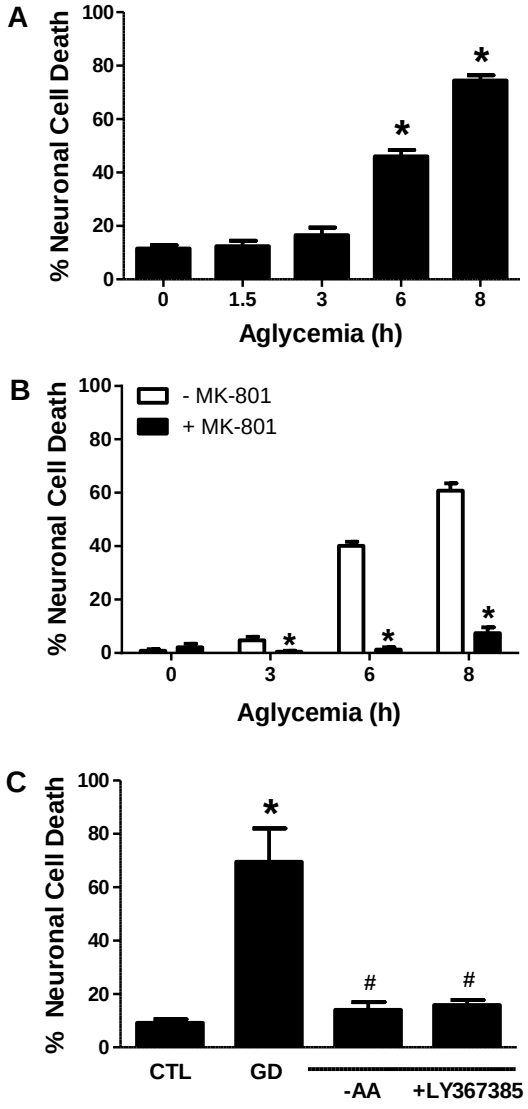

Figure 1: Aglycemic neuronal cell death is attenuated by NMDA receptor and system x_c^- antagonism. (A) Mixed cortical cultures were washed into BSS lacking or containing 10mM glucose (0 h) for the times indicated, after which neuronal cell death was determined via measurement of LDH release. An asterisk (*) represents values significantly different from control (0 h) as assessed by one-way ANOVA followed by Dunnett's post hoc test (n = 8-10). (B) Mixed cortical cultures were washed into BSS lacking or containing 10 mM glucose (0 h) ± MK-801 (10 µM). Neuronal cell death was determined via measurement of LDH release. An asterisk (*) denotes a significant between group difference as determined by two-way ANOVA followed by Bonferroni's post hoc test (n = 6). (C) Mixed cortical cultures were deprived of glucose (GD) for 8 h in a balanced salt solution containing or lacking MEM amino acids (-AA) or the dual system x_c^- /mGluR1 inhibitor, LY367365 (50 µM). Cell death was assessed immediately thereafter. An asterisk (*) represents a value significantly different from glucose containing cultures (CTL), while a (#) indicates a significant attenuation of GD-mediated neuronal cell death as assessed by one-way ANOVA followed by the Student-Newman-Keuls test (n = 8).

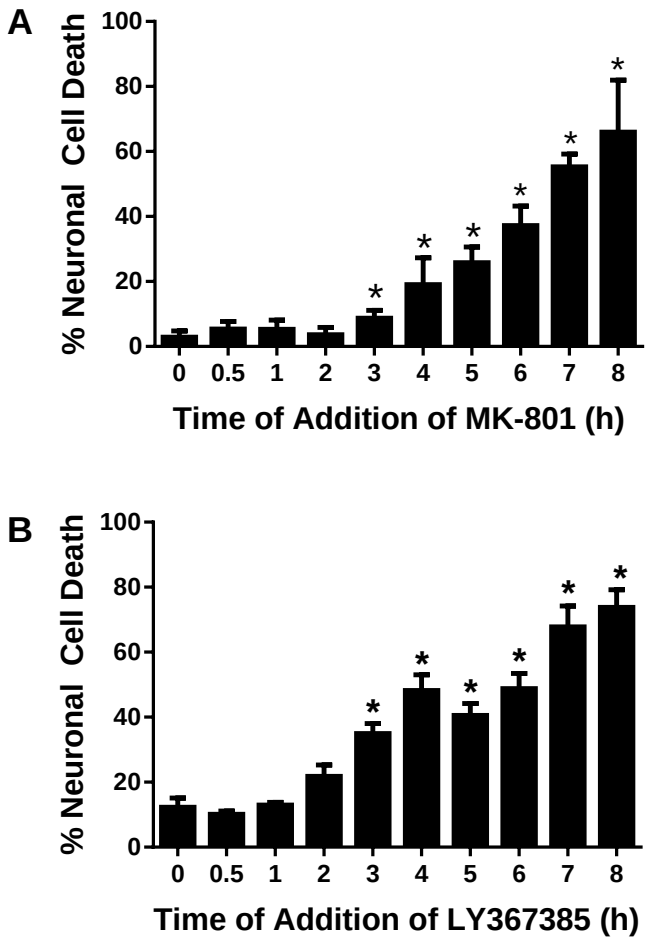

Figure 2: Neurons in mixed cortical cultures can be rescued up to two hours following the onset of glucose deprivation. Mixed cortical cultures were deprived of glucose for a total 8 h. (A) MK-801 (10 µM) or (B) LY367385 (50 µM) were added at the times indicated post GD. Asterisks (*) represent values significantly different from control cultures (0 h), which received the inhibitors at the onset of glucose deprivation (time 0). Significance was determined using one-way ANOVA and Dunnett's post hoc test for comparison to control (n = 6).

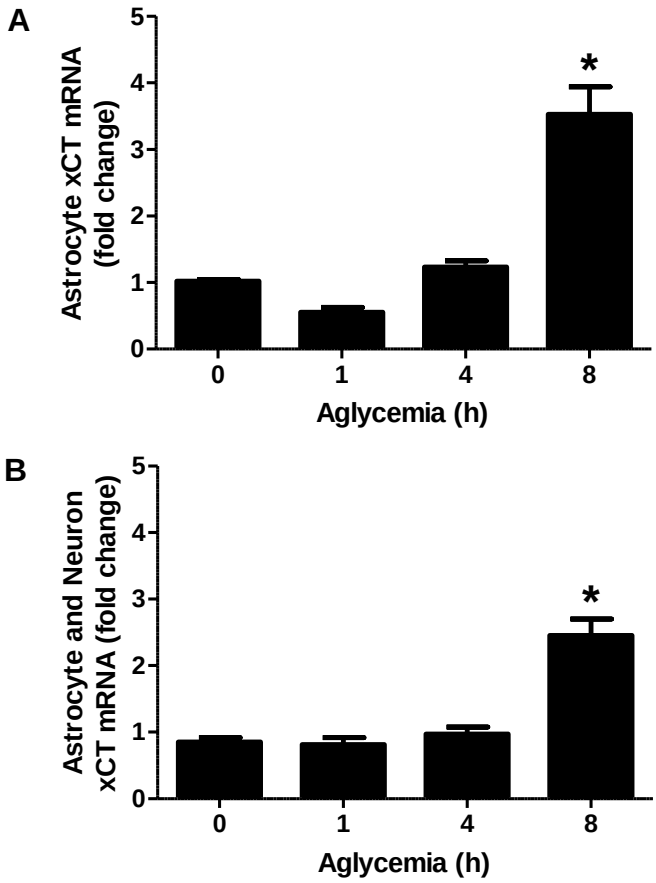

Figure 3: Glucose deprivation results in increased xCT mRNA in astrocyte and mixed cortical cell cultures. (A) Pure astrocyte cultures or (B) mixed cortical cultures containing both astrocytes and neurons (n = 4 experiments each) were deprived of glucose for the times indicated and changes in xCT mRNA assessed via qPCR. Asterisks (*) represent values that are significantly different from 0 h (i.e., glucose-containing control) as determined by one-way ANOVA followed by a Dunnett's post hoc test.

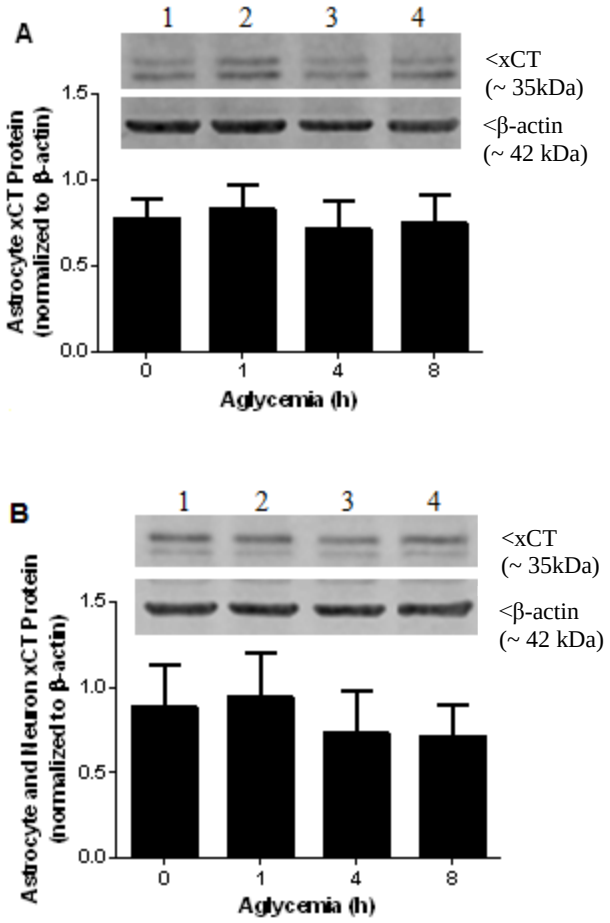

Figure 4: Glucose deprivation does not change xCT protein expression. (A) Pure astrocytes (n = 4) or (B) mixed cultures (n = 4) were washed into BSS_0 or BSS containing glucose (0 h). Cell lysates were harvested at the times indicated and 15 μg (astrocytes) or 30 μg (mixed) protein separated by SDS-PAGE. Western blot analysis was performed using a rabbit polyclonal antibody directed against xCT and a mouse monoclonal directed against β-actin. Lane 1, 0 h; Lane 2, 1 h; Lane 3, 4 h; Lane 4, 8 h. Representative of four western blots. Densitometry was performed using LICOR Image Studio software. Data are expressed as mean integrated optical density (IOD) + SEM normalized to each lane's β-actin. No significant difference from 0 h was found using one way ANOVA.

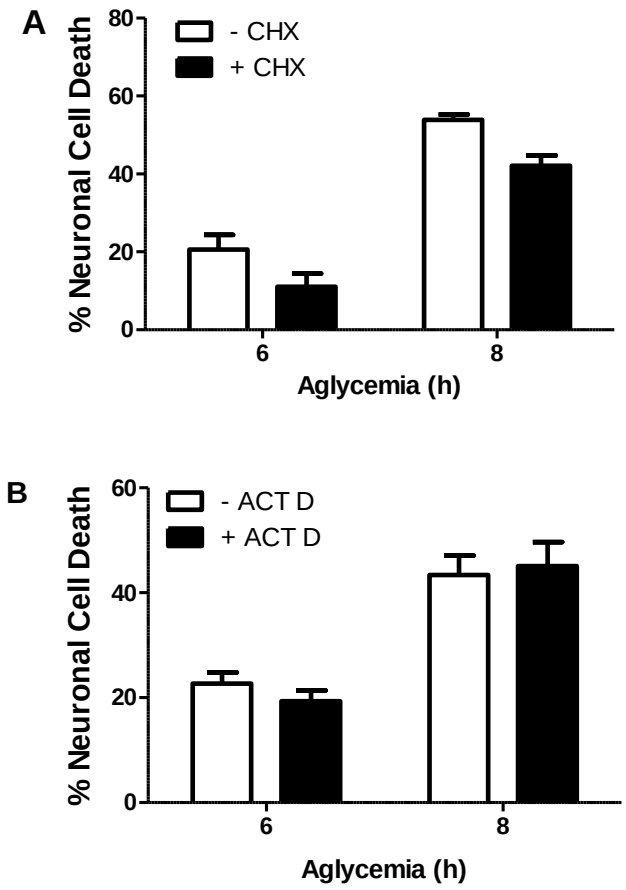

Figure 5: Glucose deprivation-induced neuronal cell death occurs independent of translation or transcription. Mixed cortical cultures were rendered aglycemic in the presence or absence of (A) the protein synthesis inhibitor cycloheximide (CHX; 1 µg/ml; n = 12) or (B) the transcriptional inhibitor actinomycin D (ACT D; 10 µg/ml; n = 6-10). Six and 8 h later, the amount of neuronal cell death was assessed. Two-way ANOVA revealed no significant between group differences in either paradigm.

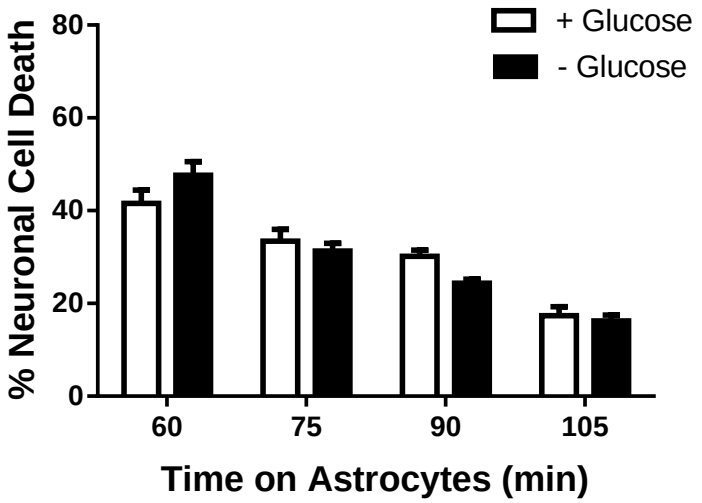

Figure 6: Glutamate uptake is not altered in glucose-deprived astrocytes. Pure astrocyte cultures were washed into BSS ± glucose for 6 h after which glutamate (final concentration = 75 µM) was added and left on the cells. At the times indicated, the cell culture medium was removed from the astrocytes, 10 mM glucose was added, and this conditioned medium was added to pure neuronal cultures. Six h later, neuronal cell death was assessed. No significant between-group differences were found using two-way ANOVA (n = 10).

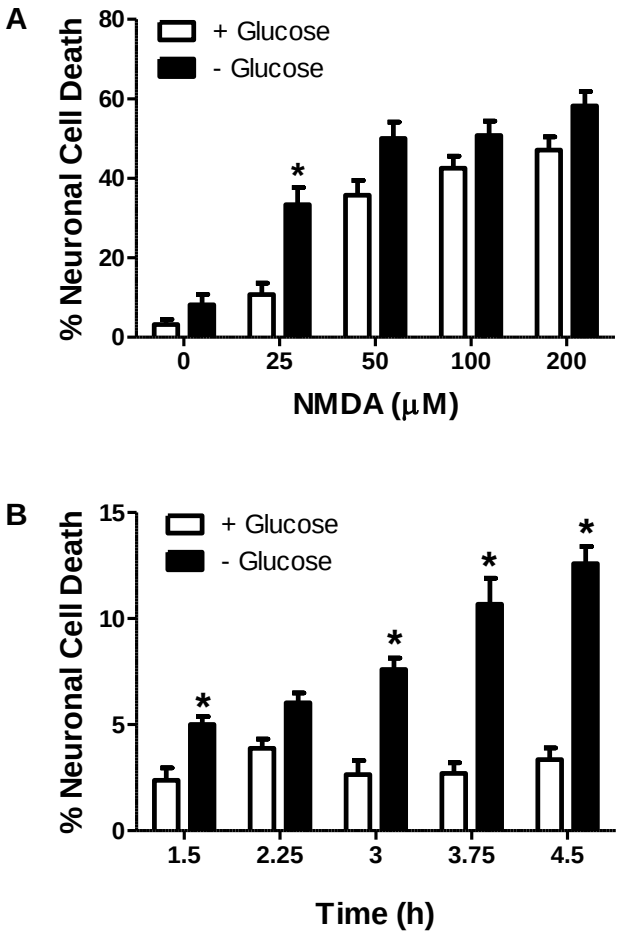

Figure 7: Glucose-deprived neurons are more susceptible to the toxic effects of NMDA. (A) Mixed cortical cultures were deprived of glucose for 2.25 h and exposed to increasing concentrations of NMDA for 10 min (n = 6) before they were washed into maintenance media. (B) Mixed cortical cultures were glucose deprived for varying times before being exposed to 25 μM NMDA (n = 8) for 10 min. Cultures were then washed into maintenance media and neuronal cell death was assessed 20-24 h later for all experiments. An asterisk (*) indicates a significant between-group difference assessed by two-way ANOVA followed by Bonferroni's post hoc test.

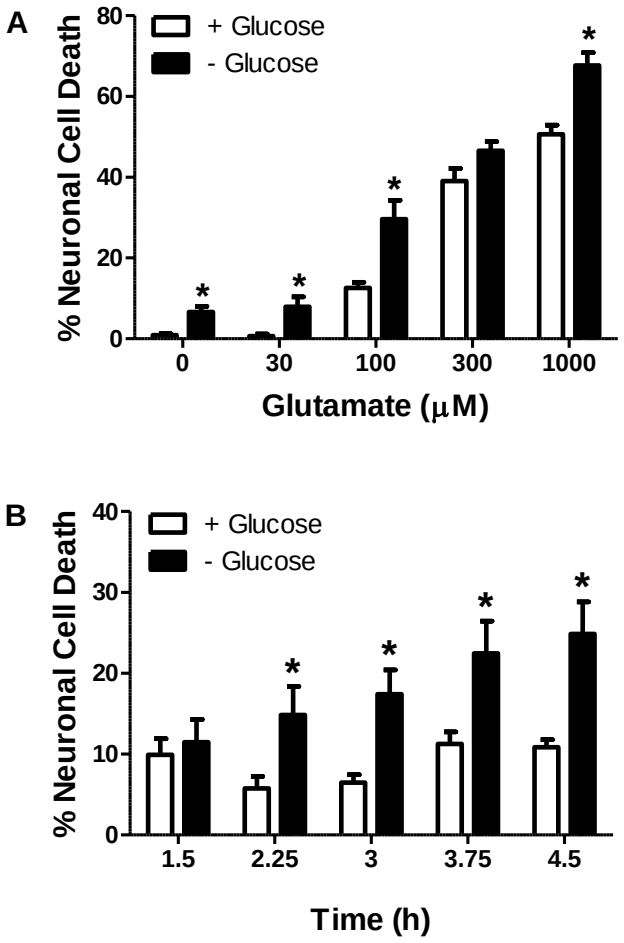

Figure 8: Glucose-deprived neurons are more susceptible to the toxic effects of glutamate. (A) Mixed cultures were exposed to increasing concentrations of glutamate for 10 min following 2.25 h of glucose deprivation (n = 6) or (B) 100 μM glutamate (n = 6) for 10 min after 1.5 to 4.5 h of glucose deprivation and assessed for neuronal cell death 20-24 h later. An asterisk (*) indicates a significant between-group difference assessed by two-way ANOVA followed by Bonferroni's post hoc test.

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