Regulation of System XC- and its Contribution to Cell Death

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by

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ABSTRACT
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The main focus of the studies in this thesis involves examining the role of cystine/glutamate exchange (system x_C-) in neuronal death in primary cortical cell culture, with an emphasis on how glial function affects neuronal cell death. System x_C- is a sodium-independent transporter that mediates cystine uptake and glutamate release. It accounts for most of the cystine uptake in astrocytes in mature cultures, providing the rate limiting substrate for synthesis of the main endogenous antioxidant glutathione. The glutamate released by system x_C- may lead to excessive extracellular glutamate and cause excitotoxicity.

β-N-methylamino-L-alanine (BMAA) is a non-protein amino acid that may be involved in neurodegenerative diseases. We found that BMAA induced oxidative stress by competing with cystine at system x_C- leading to depletion of glutathione. BMAA also drives system x_C- mediated glutamate release, which may contribute to its induction of excitotoxicity.

Fibroblast growth factor-2 (FGF-2) is involved in multiple processes in the central nervous system, including plasticity, neurogenesis, differentiation, and neuronal survival. Also, alterations in FGF-2 and its signaling have been implicated in neurodegenerative diseases and psychiatric disorders. We found that FGF-2 greatly increased cystine uptake through system x_C- in astrocyte-enriched primary cultures, but not in neuronal or microglial cultures. Our data showed that FGF-2 increased cystine uptake by upregulating system x_C- by acting on FGFR1, and signaling through the PI3K/Akt and MEK/ERK pathways.

FGF-2 treatment for 48 hours caused significant neuronal death only in mixed neuronal and glial cultures, but not in neuronal-enriched or astrocyte-enriched cultures. Blocking system x_C-, or AMPA/kainate receptors, eliminated the neuronal death induced by FGF-2 treatment. Therefore, it is likely that 48 hour FGF-2 treatment induces AMPA receptor mediated toxicity through increased glutamate release from astrocytes due to increased system x_C- function. However, we cannot exclude the possibility that FGF-2 treatment sensitizes the neurons to normal system x_C- mediated glutamate release.

Together the results indicate that 1) competitive substrates of system x_C-, such as BMAA, that do not lead to glutathione production are particularly toxic; and 2) upregulation of system x_C- on astrocytes may be toxic to surrounding neurons.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAR	amino acid response
AARE	amino acid response element
AD	Alzheimer's disease
ALS-PDC	Amyotrophic lateral sclerosis–Parkinsonism dementia complex
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
APP	amyloid-β precursor protein
ARE	antioxidant responsive element
ATF	activating transcription factor
Aβ	amyloid-β
BDNF	brain derived neurotrophic factor
BMAA	β-N-methylamino-L-alanine
BMAA	β-N-methylamino-L-alanine
BSO	buthionine sulphoximine
CHX	cycloheximide
CPG	S-4-carboxyphenyl glycine
CysGly	cysteinylglycine
DAG	diacyl glycerol
DAI	double-stranded RNA-activated inhibitor
dbcAMP	N(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate
DCF	dichlorofluorescein
EAAT	excitatory amino acid transporter
eIF2	eukaryotic initiation factor 2
EpRE	electrophile response element
ER	endoplasmic-reticulum
ERK	extracellular signal-regulated kinase
FGF-2	fibroblast growth factor 2, basic fibroblast growth factor
FGFR	FGF receptor
FRS2	fibroblast growth factor receptor substrate 2
GCN2	general control nonderepressible protein 2
GEF	guanine nucleotide exchange factor
GSH	glutathione
GSK3β	glycogen synthetase 3 β
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
HIF	hypoxia inducible factor
HRI	heme-regulated inhibitor
IGF-1	Insulin-like growth factor 1
IP3	inositol 1,4,5-trisphosphate
LPS	lipopolysaccharide
MeHg	methylmercury

MEK	mitogen-activated protein kinase kinase
MEM	memantine
mGluRs	metabotropic glutamate receptors
MK-801	[5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
MPEP	6-methyl-2-[phenylethynel]-pyridine
MPP+	1-methyl-4-phenylpyridinium ion
NASPM	1-naphthyl acetyl spermine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NCM	neuronal-conditioned media
NMDA	N-methyl-D-Aspartate
Nrf2	eythroid 2-related factor 2
NRG	neuregulin
NT-4	neurotrophin-4
O_2-	superoxide
ODAP	b-N-oxalyl-L-a,b-diaminopropionic acid
PD	Parkinson's disease
PERK	PKR-like endoplasmic-reticulum-localized eIF2α kinase
PI3-kinase	phosphatidylinositol 3 kinase
PIP2	phosphatidylinositol 4,5-bisphophate
PKA	protein kinase A
PKB, Akt	activate protein kinase B
PKC	protein kinase C
PKR	protein kinase R
PLC	phospholipase C
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative real-time PCR
Slc7a11 18	solute carrier family 7, member 11
SOD	superoxide dismutase
system X_{AG}	sodium-dependent glutamate/aspartate/cysteine transporter
system x_C-	cystine/glutamate antiporter
TGF	transforming growth factor

CHAPTER I

GENERAL INTRODUCTION

OVERVIEW

The brain is an extremely complex organ with complicated and specific interactions between multiple cell types and regions. However, neuroscience research has historically focused primarily on neurons. Glial cells have been traditionally considered as merely supporting cells. In the last couple of decades, as our knowledge of the glial cells has dramatically expanded, we now know that the function of glial cells extends far beyond just supporting neurons. Growing evidence suggests that neuronal and glial cells communicate through neurotransmitters, neuromodulators, and growth factors, and this bidirectional communication is critical for normal function of the brain.

The aim of this thesis is to explore the interaction between neurons and glia, and how that interaction regulates neuronal fate, with a particular emphasis on the involvement of the cystine/glutamate antiporter (system x_C^-) (Fig. 1.1). System x_C^- is mainly expressed on astrocytes and it is the main route of cystine uptake in these cells. This cystine uptake is the critical step in synthesizing the major antioxidant glutathione. Astrocytes then release glutathione, and other cysteine containing molecules, to supply neurons with cysteine for them to produce glutathione. With every molecule of cystine uptake into the astrocytes, one molecule of glutamate is exchanged out of the cell. This extrasynaptic, nonvesicular, release of glutamate not only serves to regulate synaptic function, but also when excessive, can over-activate glutamatergic receptors on the neurons to cause excitotoxicity. Both oxidative stress and excitotoxicity have been implicated in various neurodegenerative diseases, as well as psychiatric disorders. Thus, it is possible that system x_C^- plays a role in these diseases.

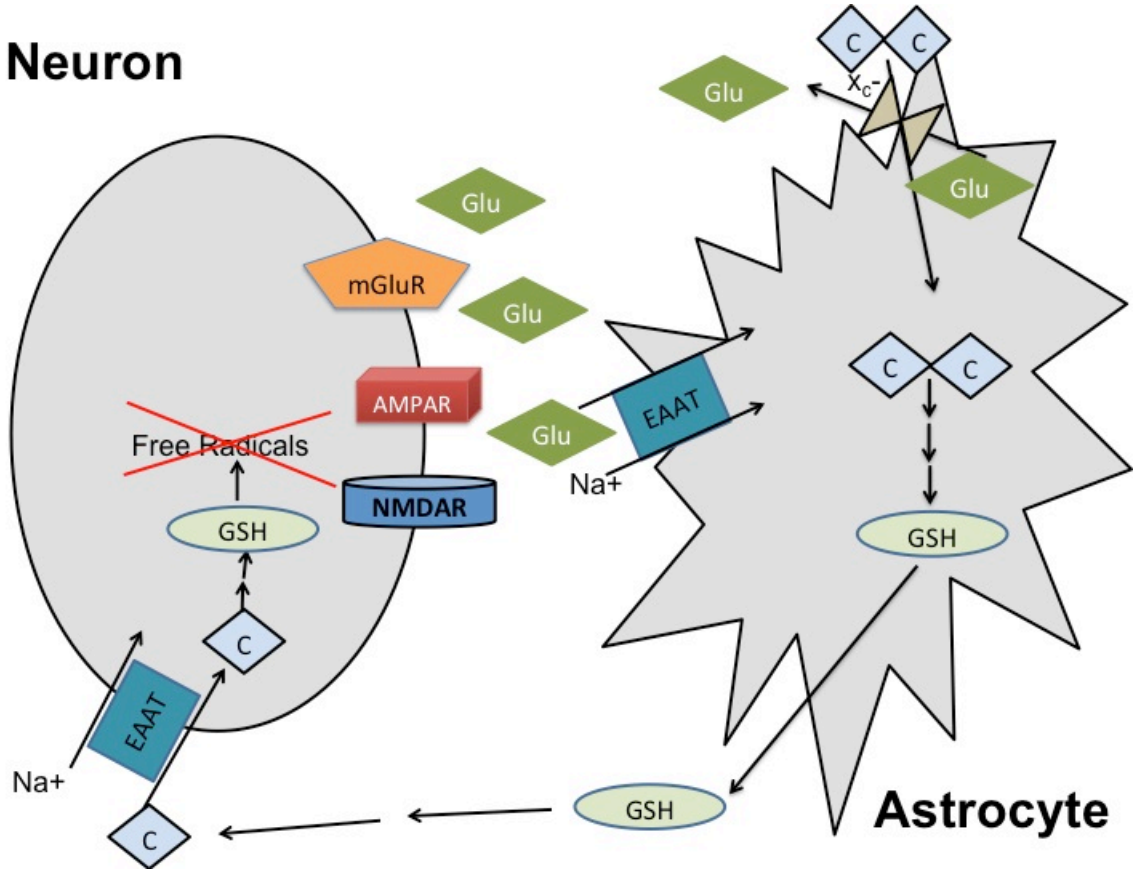

FIGURE 1.1. Schematic diagram illustrating the function of system x_C^- and the interaction between neuron and astrocyte.

C: cysteine, CC: cystine, Glu: glutamate, GSH: glutathione, EAAT: excitatory amino acid transporter

Several pathways that are involved in system x_C^- regulation have been identified. However, the effects of changing system x_C^- function are not well understood. Since targeting system x_C^- as a treatment for various brain disorders is being considered, it is very important to achieve a full understanding of system x_C^- function and regulation, as well as the consequences of changing its activity.

GLUTAMATE NEUROTRANSMISSION

Glutamate is the most important excitatory neurotransmitter in the brain (Fonnum, 1984). It is also a precursor for the most important inhibitory neurotransmitter GABA (Petroff, 2002; Schousboe and Waagepetersen, 2007), and is a component of glutathione, one of the major antioxidants in the brain (Meister and Anderson, 1983; Dringen et al., 2000). Glutathione can be synthesized de novo from glucose in astrocytes via the Krebs cycle, leading to the release of glutamine from astrocytes, which is taken up by neurons and hydrolyzed into glutamate by glutaminase (Erecińska and Silver, 1990). Glutamate can activate a large family of receptors existing on neurons and astrocytes leading to signal transmission.

Glutamate receptors

Glutamate signal transmission is mediated by release of glutamate and activation of various glutamatergic receptors. Generally, as illustrated in Fig. 1.2, glutamatergic receptors can be divided into ionotropic receptors and metabotropic receptors. Ionotropic receptors include N-methyl-D-Aspartate (NMDA) receptors and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA)/kainate receptors, while metabotropic glutamate receptors (mGluRs) can be further divided into three subgroups. Specific receptors and their properties are briefly described below (for a detailed review see (Dingledine et al., 1999)).

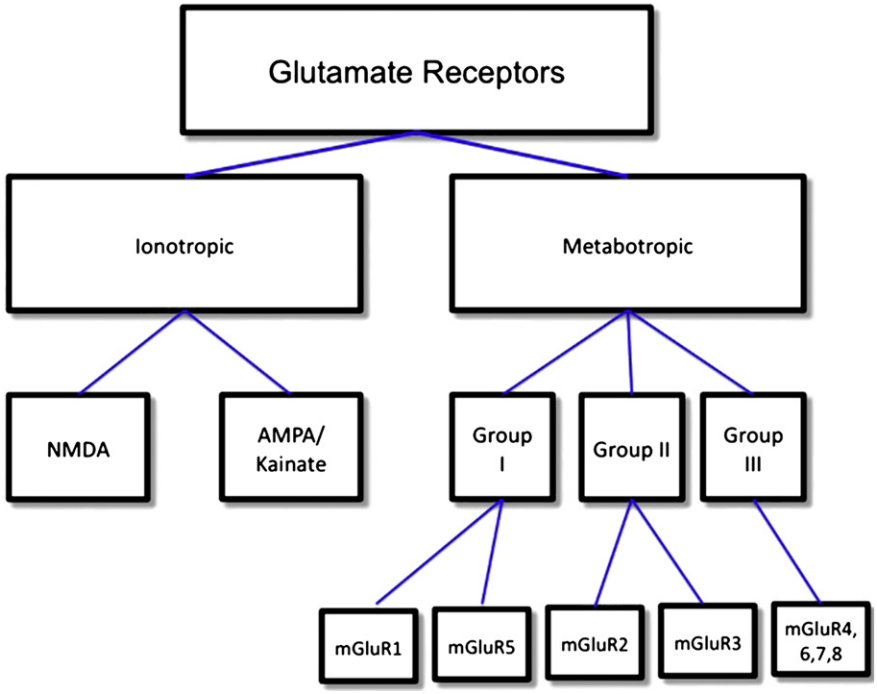

FIGURE 1.2. Glutamate receptor subtypes
(From (Niciu et al., 2012))

AMPA/Kainate receptors

AMPA receptors are another family of glutamatergic receptors that are characterized by low affinity (EC50 ~ 500 μM) (Lomeli et al., 1994; Mosbacher et al., 1994; Schoepfer et al., 1994). AMPA receptors are heterotetrameric receptors composed of four subunits (GluR1-4) (Alt et al., 2004). Unlike the other GluRs, GluR2 has a Q/R site (uncharged glutamine/positively charged arginine) located in the pore of the channel. Physiologically almost all of the GluR2 subunits are edited to the R form, which makes the receptor impermeable to calcium (Jonas et al., 1994; Petroff, 2002; Schousboe and Waagepetersen, 2007). Therefore, while all AMPA receptors are permeable to sodium and potassium, GluR2 lacking AMPA receptors are also calcium permeable (Meister and Anderson, 1983; Dringen et al., 2000; Clem and Barth, 2006), which awards these

receptors the important property of increasing intracellular calcium levels when they are activated (Erecińska and Silver, 1990; Schneggenburger et al., 1993).

Kainate receptors are tetramers of GluR5, GluR6, GluR7, KA1, and KA2 (Dingledine et al., 1999). Similar to AMPA receptors, they are ion channels that are permeable to sodium and potassium (Dingledine et al., 1999; Niciu et al., 2012). However, the functions of kainate receptors are not well defined.

NMDA receptors

NMDA receptors are another important ionotropic receptor subtype that are usually heteromers of GluN1 and GluN2 (GluN2A, GluN2B) subunits in mature brain (Béhé et al., 1995; Premkumar and Auerbach, 1997). Compared to AMPA receptors, NMDA receptors are characterized by a high affinity (EC_{50} ~1 μM) for glutamate (Patneau and Mayer, 1990; Burnashev et al., 1995). However, under physiological conditions, they are normally blocked by magnesium at negative membrane potential (Nowak et al., 1984). During normal synaptic activity, AMPA receptors must be activated first to depolarize the cell membrane, which removes the magnesium block (Nowak et al., 1984; Dingledine et al., 1999). NMDA receptor activation also requires binding of a co-agonist, such as glycine or D-serine (Johnson and Ascher, 1987; Schell et al., 1995). NMDA receptors are nonspecific cation channels that are permeable to sodium, potassium, and calcium (Grienberger and Konnerth, 2012), with the permeability to calcium distinguishing them from most AMPA/Kainate receptors.

mGluRs

mGluRs are G-protein coupled receptors that exist on neurons and glial cells (Conn and Pin, 1997; Ferraguti and Shigemoto, 2006; Kim et al., 2008). Like all G-protein coupled receptors, mGluRs have 7 transmembrane spanning domains, an agonist binding domain (N-terminus), as well as an intracellular domain (C-terminus) that couples to different G-proteins (Niciu et al., 2012). To date, there are 8 known family members as illustrated in Fig. 1.2: mGluR1-8. They are divided into three subfamilies: Group 1 (mGluR1 and 5) that are coupled to G_q, Group II (mGluR2 and 3) and Group III (mGluR 4, 6, 7 and 8) that are coupled to G_i. Activation of G_q leads to activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphophate (PIP2) to secondary messengers diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which then lead to the increase in intracellular free calcium levels and activation of calcium dependent protein kinases such as protein kinase C (PKC) (Miller et al., 1995; Conn and Pin, 1997). On the other hand, activation of G_i leads to inhibition of adenylyl cyclase, decreased cAMP, and decreased activation of protein kinase A (PKA) (Winder and Conn, 1993; Niciu et al., 2012). Group I mGluRs are mainly localized to post synaptic and glial membranes, while the majority of Group II mGluRs are localized to presynaptic membranes and provide an autoinhibition mechanism for neurotransmitter release.

Excitotoxicity

Although glutamate transmission is essential to normal brain function, excessive extracellular glutamate can cause excitotoxicity. Excitotoxicity is typically caused by

over-activation of glutamatergic receptors, especially NMDA receptors due to their permeability to calcium and slower inactivation, leading to excessive calcium influx into the cells to trigger cell death (Choi, 1987). This type of neuronal death can occur in conditions such as stroke, traumatic brain injury, and neurodegenerative diseases (Bains and Shaw, 1997; Choi, 1998).

In certain situations, over-activation of AMPA receptors can cause excitotoxicity as well. AMPA receptor over-activation by addition of AMPA, a direct agonist for AMPA receptors, has been shown to be toxic to oligodendrocytes (McDonald et al., 1998). The general AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) has been shown to be protective to oligodendrocytes in multiple sclerosis models (Pitt et al., 2000; Smith et al., 2000). Oligodendrocyte toxicity often appears to be mediated by calcium permeable GluR2 lacking AMPA receptors because it is prevented by the selective antagonist 1-naphthyl acetyl spermine (NASPM) (Yoshioka et al., 1996; Bannerman et al., 2007).

Regulation of extracellular glutamate by astrocytic glutamate transporters

Total glutamate concentration is extremely high in the brain (10 mM) (Erecińska and Silver, 1990), but the extracellular glutamate concentration is very low (below 10 μM) (Ronne-Engström et al., 1995; Baker et al., 2003; Rodriguez et al., 2012). The extracellular glutamate is tightly regulated by the excitatory amino acid transporters (EAATs). EAATs mediate sodium-dependent high-affinity glutamate uptake, which is driven by the sodium concentration gradients: for every glutamate taken up, three molecules of sodium enter the cell while one molecule of potassium exits the cell

(Barbour et al., 1988; Zerangue and Kavanaugh, 1996a). Astrocytes with abundant glutamate transporters EAAT1/GLAST and EAAT2/GLT-1 activity-dependently ensheath glutamatergic synapses (Ventura and Harris, 1999; Witcher et al., 2010), where they are involved in clearing extracellular glutamate to avoid unwanted prolonged synaptic activation and excitotoxicity (Amara, 1992; Kanai and Hediger, 1992; Storck et al., 1992; Rothstein et al., 1994; 1996; Diamond and Jahr, 1997; Lehre and Danbolt, 1998). Glutamate taken up by astrocytes is converted into glutamine by glutamine synthetase. Glutamine, in turn, can be released and taken up by neurons to synthesize glutamate. This glutamate-glutamine cycle between neurons and astrocytes ensures signaling specificity and a fast turnover rate (Sibson et al., 1998).

Synaptic and extrasynaptic compartments

Another important function of astrocytes ensheathing synapses is that they spatially separate synaptic and extrasynaptic compartments of the extracellular space. Astrocytic EAATs provide efficient buffering and clearance of glutamate to prevent spillover and cross-talk between different synapses and compartments (Rothstein et al., 1996; Asztely et al., 1997; Rusakov and Kullmann, 1998).

Synaptic and extrasynaptic receptors seem to have different functions; the most studied example are the synaptic and extrasynaptic NMDA receptors. It has become an increasingly accepted point of view that synaptic NMDA receptor activation is neuroprotective, while extrasynaptic NMDA receptor activation turns off the neuroprotective synaptic NMDA receptor activation and also activates intracellular pathways that lead to neuronal death (Hardingham et al., 2002; Riccio and Ginty, 2002;

Ivanov et al., 2006; Léveillé et al., 2008; Xu et al., 2009; Hardingham and Bading, 2010). Drugs that preferentially block extrasynaptic NMDA receptors have received particular attention as potential treatments for neurodisorders. For example, memantine (MEM), which is an FDA approved drug for late stage Alzheimer's disease, has been shown to have selectivity for extrasynaptic NMDA receptors (Lipton and Chen, 2004; Xia et al., 2010). MEM is an open channel blocker at NMDA receptors. At low doses, it does not accumulate in the synaptic cleft to interfere with synaptic NMDA receptor mediated signaling. However, it does antagonize extrasynaptic NMDA receptors that are hyperactive due to increased extrasynaptic glutamate levels in diseased brains (Lipton and Chen, 2004; Chen and Lipton, 2006; Xia et al., 2010).

NEURON-GLIA INTERACTION

Bidirectional communication between astrocytes and neuronal cells is necessary for the normal functioning of the nervous system during signal processing. Some interactions between neurons and glia are discussed below with a focus on a typical glutamatergic synapse as shown in Fig. 1.3.

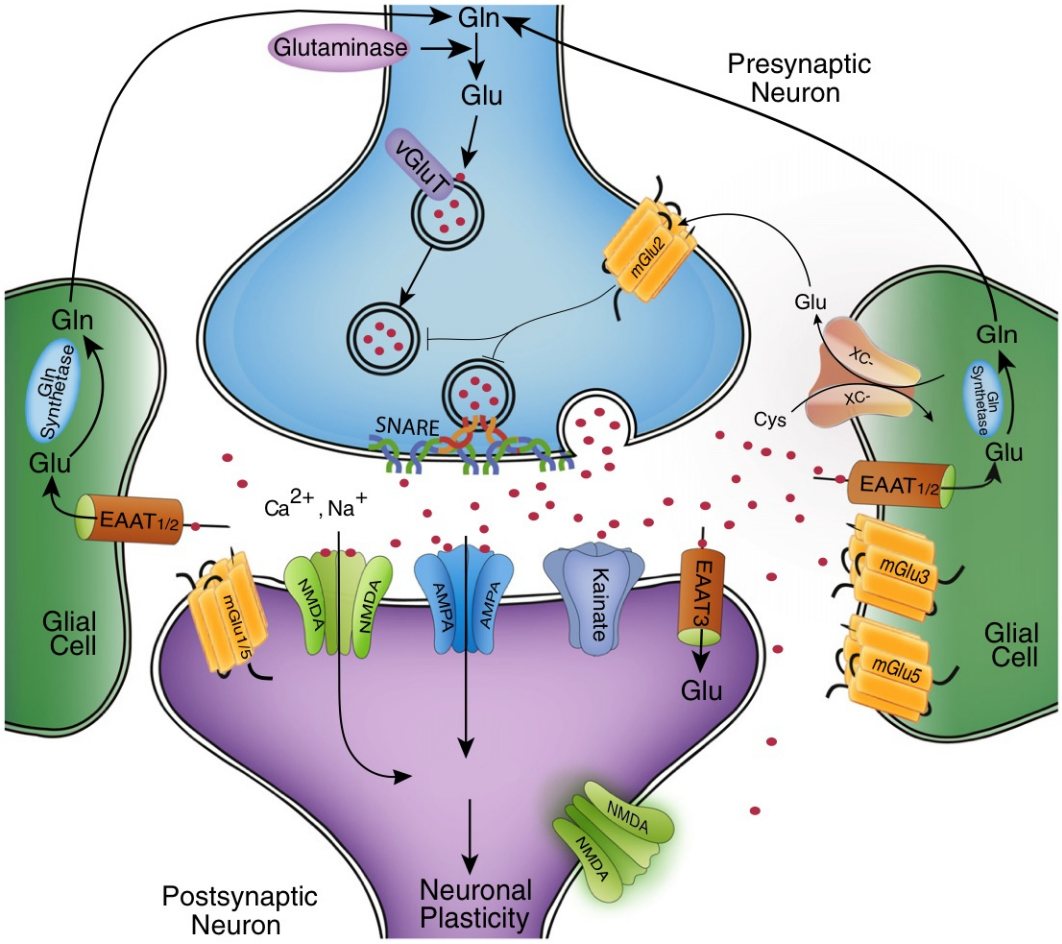

FIGURE 1.3. A typical glutamate synapse.
(Altered From (Niciu et al., 2012))

Regulation of astrocytes by neurons

Physiologically, high levels of neuronal activation can cause elevated extracellular potassium levels, and in turn, slowly depolarize glial cells (Meeks and Mennerick, 2007; Sasaki et al., 2011). Under extreme conditions, depolarization can spread throughout the astrocyte network and cause cortical spreading depression (Unekawa et al., 2012; Bogdanov et al., 2013).

Similar to neurons, astrocytes also express a variety of receptors to respond to neurotransmitter release, such as mGluRs (Venance et al., 1997), nicotinic acetylcholine receptors (Oikawa et al., 2005), adrenoceptor , P2 receptors of the P2X (ligand-gated cationic channels) and P2Y (G-protein coupled receptors) types (Butt, 2011; Köles et al., 2011). Most of these astrocytic receptors are G_q-protein coupled receptors. Unlike neurons, astrocytes do not generate action potentials, However, activation of these astrocytic G_q-protein coupled receptors by neurotransmitters can lead to elevated intracellular calcium levels (Venance et al., 1997; Agulhon et al., 2008). Astrocytes also express some ionotropic glutamate receptors (Seifert and Steinhäuser, 2001), although their functional significance is largely unknown.

Regulation of neurons by astrocytes

Each astrocyte is believed to have its own territory, and within that territory, it may interact with 140,000, or more, synapses and neuronal processes (Benarroch, 2009). Therefore, individual astrocytes are potentially capable of coordinating a large amount of neuronal activity (Poskanzer and Yuste, 2011). Astrocytes are also connected to each other through gap junctions, which allows fast chemical and electrical communication among astrocytes, enabling them to function as a network. Thus, activation of a single astrocyte can spread to an extended surrounding area and potentially regulate the function of multiple neighboring neurons (Cornell-Bell et al., 1990).

Astrocytes can also release gliotransmitters (such as glutamate, ATP, D-serine etc.) to regulate neuronal function and synaptic plasticity (Fellin et al., 2006; Butt, 2011; Parpura et al., 2012). It is known that increased calcium levels in astrocytes, like that in

neurons, can trigger the fusion of vesicles containing gliotransmitters with the plasma membrane (Bezzi et al., 1998; 2004; Kreft et al., 2004). Non-vesicular release of glutamate has also been suggested. A recent study describes a channel mediated release of gliotransmitter (<900 Da), such as glutamate (Duan et al., 2003). System x_C^- mediates a selective non-vesicular release of glutamate. It is believed that glutamate released from astrocytes mainly activates the extrasynaptic pool of glutamatergic receptors (Xi et al., 2002).

Astrocyte and neuron metabolic coupling

As the largest population of cells in the nervous system, astrocytes are crucial in maintaining normal glutamate transmission (Ye and Sontheimer, 2002; Huang et al., 2004; López-Bayghen and Ortega, 2011). Glucose is the major substrate for brain energy production. Astrocytes take up glucose from their endfeet on capillaries (Magistretti and Pellerin, 1996; Edvinsson and Krause, 2002), metabolize it, and release L-lactate and pyruvate as energy sources for neurons (Dringen et al., 1993). Metabolites from glucose can also be further processed to produce essential neurotransmitters such as GABA and glutamate (Magistretti and Pellerin, 1996). Glutamate uptake and glucose utilization by astrocytes are tightly coupled as illustrated in Fig 1.4.

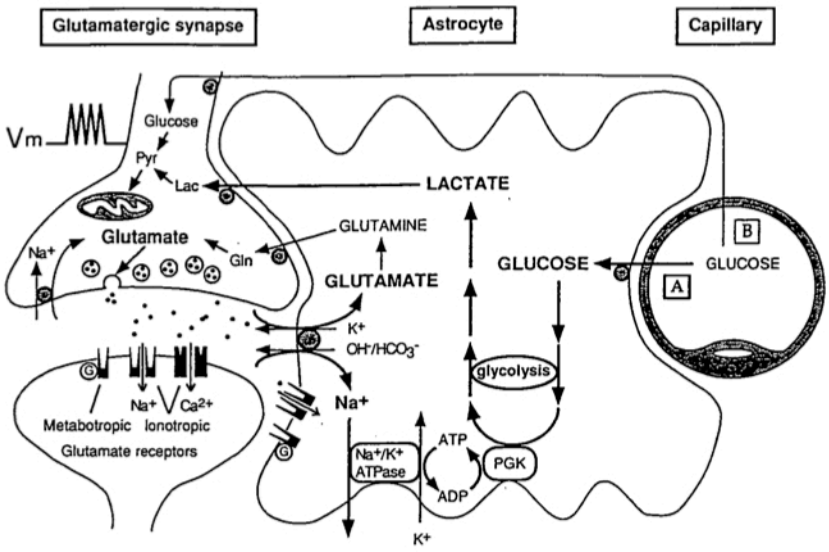

FIGURE 1.4. Schematic representation of glutamate cycling and glucose metabolism coupling.

At glutamatergic synapses, excessive extracellular glutamate is removed by a glutamate uptake system located primarily on astrocytes. Glutamate is cotransported with Na^+, resulting in an increase in the intracellular concentration of Na^+ in astrocytes leading to activation of the Na^+/K^+ ATPase pump. The pump utilizes ATP, which is provided by membrane-bound glycolytic enzymes. This demand for ATP activates glycolysis in astrocytes, resulting in the production of lactate. Lactate, once released can be taken up by neurons and serve as an energy substrate. From (Magistretti and Pellerin, 1996)

OXIDATIVE STRESS AND GLUTATHIONE IN THE BRAIN

Oxidative stress

Oxidative stress is an excessive accumulation of free radicals and other reactive oxygen species (ROS). It can be caused by either an overproduction of free radicals or a deficit in their clearance. ROS are normal products of cellular metabolism. While some levels of free radical generation is normal and necessary, overwhelming amounts of free radicals can lead to oxidative damage to proteins, lipids and DNA, causing dysfunction of these molecules (Freeman and Crapo, 1982; Simonian and Coyle, 1996). As illustrated in Fig. 1.5., ROS are mainly generated in the mitochondria: electrons along the electron

transport chain in mitochondria leak out and are accepted by oxygen resulting in superoxide (O_2^-) production (Freeman and Crapo, 1982). There are various cellular defense mechanisms against ROS, such as superoxide dismutase (SOD), catalase, glutathione (GSH), vitamin C and vitamin E (Sies, 1997; Brigelius-Flohé and Traber, 1999). SOD can degrade superoxide generating hydrogen peroxide (H_2O_2) (Fridovich, 1975). H_2O_2 is not reactive. However, when there are metal ions present, highly reactive hydroxyl radical can be generated by Fenton reaction. In contrast, catalase can decompose H_2O_2 into water and oxygen. The H_2O_2 can also be eliminated by GSH in a process mediated by glutathione peroxidase, generating glutathione disulfide (GSSG). GSSG can be reduced back to GSH by NADPH via glutathione reductase (Fig.1.5). Besides reducing oxidative stress, GSH can also directly conjugate to toxins, and both GSH and GSH conjugates can be transported out of the cells by multi-drug resistance proteins (Rush et al., 2012b).

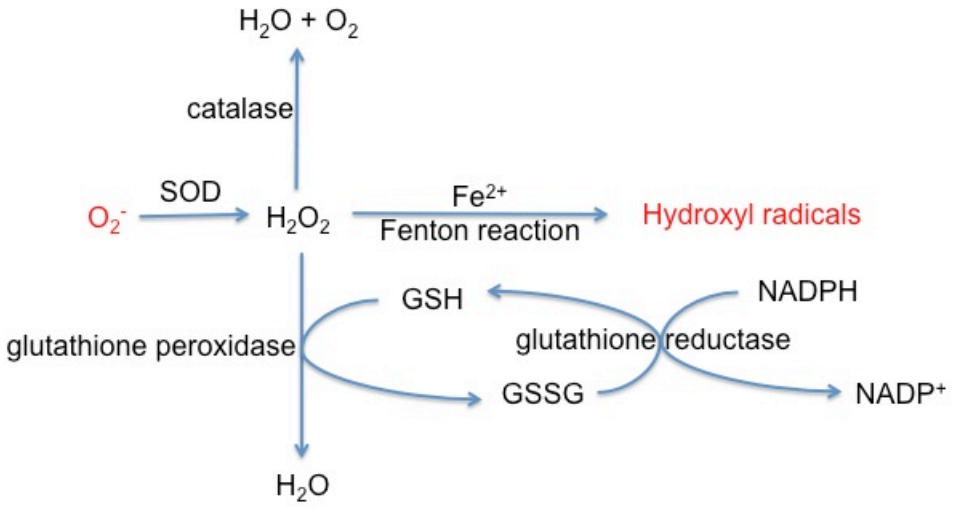

FIGURE 1.5. Diagram illustrating the different pathways for handling O_2^-.
O_2^-: superoxide, SOD: superoxide dismutase, GSH: glutathione, H_2O_2: hydrogen peroxide, GSSG: glutathione disulfide.

The brain, while only 2% of the body weight, consumes about 20% of the total oxygen, generating high levels of ROS (Ballatori et al., 2009), and oxidative stress has been shown to be a possible contributor to the damage occurring in neurodegenerative diseases (Simonian and Coyle, 1996; Schulz et al., 2000; Ballatori et al., 2009).

GSH in the brain

GSH is a tripeptide consisting of the amino acids glutamate, glycine, and cysteine (Dringen et al., 2000). It is the most prevalent cellular thiol in the brain, with an intracellular concentration of ~2.5 mM in neurons and ~ 3.8 mM in astrocytes (Bolaños et al., 1995; Rice and Russo-Menna, 1998). Normally, the intracellular GSSG/GSH ratio is tightly regulated, with over 99% of the total cellular GSH present as the reduced form (Deneke and Fanburg, 1989). The GSSG/GSH ratio is a sensitive indicator of oxidative stress (Rahman et al., 2005).

GSH is synthesized via a two-step reaction (Fig. 1.6) (Beutler, 1989; Deneke and Fanburg, 1989). First, glutamate and cysteine are catalyzed to γ-glutamylcysteine by glutamate cysteine ligase. Then glycine joins γ-glutamylcysteine mediated by glutathione synthetase. Both steps require ATP. Both glutamate and glycine are highly available in the cells, so the rate-limiting factor is cysteine. The rate of this reaction is based on intracellular cysteine levels and γ-glutamylcysteine synthetase that is feedback-regulated by GSH. Inhibiting cystine uptake inhibits GSH synthesis because the levels of intracellular cysteine are dependent on cystine uptake (Bannai and Kitamura, 1980).

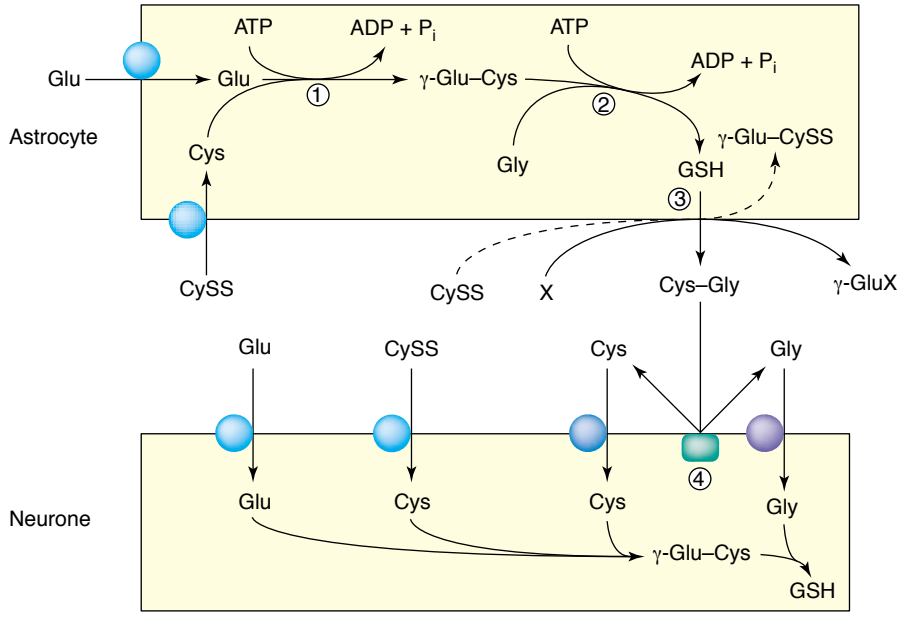

FIGURE 1.6. GSH synthesis and cycling in the central nervous system.

In astrocytes: 1: γ -glutamylcysteine synthetase; 2: glutathione synthetase; 3: γ -glutamyl transpeptidase. In Neurons: 4: dipeptidases. Glu: glutamate, CySS: cystine, Cys: cysteine, Gly: glycine, GSH: glutathione. From (McBean, 2002)

Sources for cysteine seem to depend on the developmental stage and cell type in the central nervous system (Kranich et al., 1996). It is believed that the sodium-independent system x_C^- is the main mechanism of cystine uptake in glial cells (Lobner, 2009), while the sodium-dependent glutamate/aspartate/cysteine transporter [System X_{AG} (EAAT3/EAAC1)] is responsible for most of cysteine uptake in mature neurons (McBean, 2002). Cystine will immediately be broken down by thioredoxin reductase 1 into two cysteine molecules once it enters the cells (Arrick et al., 1985).

In the nervous system, glial cells are important in supplying neurons with cysteine containing molecules, such as cysteine itself, cysteinylglycine (CysGly), or GSH (Dringen et al., 1999). Extracellularly, GSH is metabolized by the ectoenzyme γ-glutamyl transferase, which transfers the γ-glutamyl residue of GSH to different acceptor

amino acids leading to the formation of a γ-glutamyl containing dipeptide and a dipeptide CysGly. CysGly is then either cleaved by extracellular dipeptidases to generate cysteine and glycine, or directly taken up by neurons. Knocking out EAAT3 greatly reduces neuronal cysteine uptake and intracellular GSH levels, resulting in decreased viability of hippocampal neurons against H_2O_2 insults (Zerangue and Kavanaugh, 1996b; Chen and Swanson, 2003; Aoyama et al., 2006). This suggests that EAAT3 plays a critical role in the ability of neurons to obtain cysteine.

SYSTEM XC-

System x_C^- is a sodium-independent, chloride-dependent amino acid transport system located on the plasma membrane. System x_C^- was first characterized by Bannai and Kitamura in 1980 when mutual inhibition of glutamate and cystine uptake was described (Bannai and Kitamura, 1980). It can transport one molecule of cystine into the cell in exchange for one molecule of intracellular glutamate, with K_m values of ~ 80 μM for cystine uptake and ~ 160 μM for glutamate uptake (Sato et al., 1999). The transport direction is determined by the high cytosolic glutamate levels and low cytosolic cystine levels.

Structure of system x_C^-

System x_C^- is a heteromeric antiporter, composed of two subunits: a light-chain subunit xCT and a heavy chain 4F2hc, which are linked together by one disulfide bond (Torrents et al., 1998; Shih et al., 2006) (Fig. 1.7.). The 4F2hc subunit is a single transmembrane glycoprotein that is believed to be universally shared among the

heterodimer membrane transporter family: transporter systems L (Lewerenz et al., 2012; Bridges et al., 2012a). Although 4F2hc by itself does not have amino acid transport function, it is essential to system x_C^- function because it brings xCT to the membrane (Estévez et al., 1998; Sato et al., 1999; Bassi et al., 2001). xCT determines the substrate specificity and efficiency of system x_C^-. It is encoded by the solute carrier family 7, member 11 (Slc7a11) gene (Bassi et al., 2001), which produces a 502 amino acid short chain protein with a predicted molecular mass of about 55.5 kDa (Sato et al., 1999). The xCT subunit is highly hydrophobic and has a 12 transmembrane domain with a re-entrant loop and both N- and C-termini are located on the cytoplasmic side (Gasol et al., 2004).

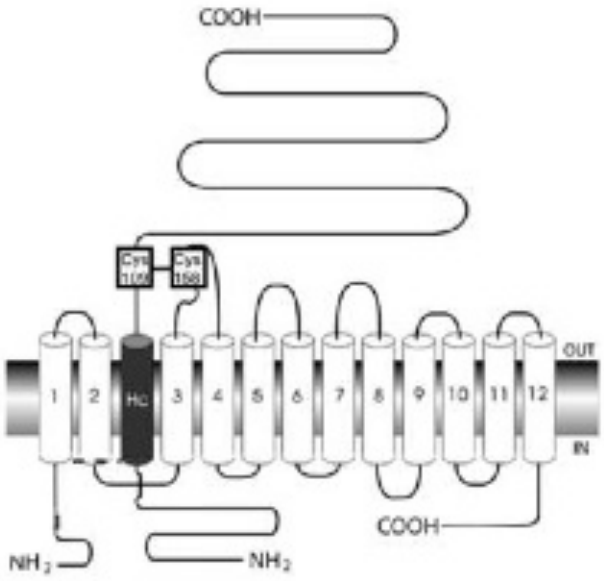

FIGURE 1.7. The structure of system x_C^-

System x_C^- is composed of a light chain xCT (shown in clear) and a heavy chain 4F2hc (shown in black), connected by a single disulfide bridge (shown in squares). From (Shih et al., 2006).

Function of system x_C-

Because of the dual transport property of system x_C-, it can regulate both intracellular GSH levels and extracellular glutamate levels. Cystine that enters cells through system x_C- can be broken down to cysteine to synthesize GSH, which can reduce free radicals or be released to regulate the redox state of the extracellular millieu (Wang and Cynader, 2000; Banjac et al., 2008). System x_C- has been shown to be responsible for 60% of extracellular glutamate in rat striatum (Baker et al., 2002). Deleting the xCT gene in Drosophila causes a 50% reduction in extracellular glutamate levels (Augustin et al., 2007). Extracellular glutamate released from system x_C- can activate presynaptic mGluR2/3, which can regulate synaptic release of neurotransmitters, such as dopamine (Baker et al., 2002).

Sato et al. 2005, developed and characterized xCT null mice with a partial deletion of the xCT gene. These mutant mice appear healthy and fertile. However, in plasma, the cystine concentration is doubled compared to wild type, while GSH levels are half of the wild type (Sato et al., 2005). No difference in cysteine levels was reported. Microglial cells isolated from these mice showed normal levels of cystine uptake. However, the uptake was not blocked by glutamate and was not inducible by lipopolysaccharide (LPS) (Sato et al., 2005). Fibroblasts isolated from these xCT -/- mice die unless exogenous 2-mercaptoethanol or N-acetylcysteine, which reduces cystine to cysteine, is present (Sato et al., 2005). In xCT deficient mice, ischemia-reperfusion-induced acute renal failure is more severe compared to wild type animals (Shibasaki et al., 2009). No increased oxidative stress or brain atrophy were observed (De Bundel et al., 2011). This is probably because in xCT deficient animals other cystine/cysteine

uptake transporters are upregulated to maintain the normal activity. However, it is not possible to upregulate in these xCT deficient animals under oxidative stress to help increase GSH levels (Shibasaki et al., 2009). Therefore, it appears that some degree of compensation for the lack of system x_C^- occurs, but it is not entirely effective.

Another mutant mouse line involving altering system x_C^- function is the sut/sut mouse, which has a partial deletion of the xCT gene. These animals show changes in fur color due to a deficiency in the cysteine-dependent yellow/red pigment, pheomelanin (Chintala et al., 2005), and a large reduction in pheomelanin is also observed in cultured sut/sut melanocytes. Interestingly, sut/sut mice also exhibit prominent brain atrophy in the hippocampus (Shih et al., 2006). The mechanisms resulting in different phenotypes of these two different mutant mouse lines require further studies.

Regulation of system x_C^-

Despite the involvement of system x_C^- in both excitotoxicity and oxidative stress, there is limited knowledge about its regulation. Because xCT is specific to system x_C^- and determines specificity of its transport function, the regulation studies have been mainly focused on xCT regulation (Sato et al., 1999). To date, the best-characterized pathways are nuclear factor eythroid 2-related factor 2 (Nrf2)-antioxidant responsive element (ARE) and eukaryotic initiation factor-2 (eIF2) - activating transcription factor (ATF) 4- amino acid response element (AARE), which are discussed in detail below, along with other regulatory mechanisms.

Nrf2-ARE pathway

There are four ARE-like, also known as electrophile response element (EpRE)-like, sequences in the 5' flanking region of the mouse xCT gene (Sasaki et al., 2002), two of which are completely conserved in the 5'-flanking region of the human xCT gene (Sato et al., 2000; Sasaki et al., 2002).

The transcription factor Nrf2-ARE pathway was first proposed by Venugopal and Jaiswal (Venugopal and Jaiswal, 1996), as illustrated in (Fig. 1.8). Nrf2 is normally cytosolic because of keap-1 binding (Itoh et al., 1999). However, when under oxidative stress, Nrf2 is freed and translocates to the nucleus and binds to ARE to activate specific protein transcription (Itoh et al., 1999). ARE is a cis-acting regulatory element located in the promoter regions of multiple genes encoding phase II detoxification enzymes and antioxidant proteins (Lee and Johnson, 2004). These proteins include heme oxygenase, γ-glutamylcysteine synthetase, glutamate-cysteine ligase, glutathione synthetase, glutathione S-transferase, glutathione reductase, multidrug resistance protein 1, as well as xCT (Bannai, 1984; Erickson et al., 2002; Sasaki et al., 2002).

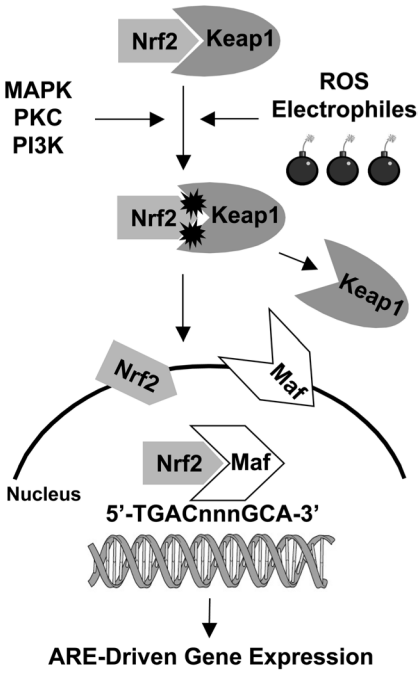

FIGURE 1.8. Nrf2-ARE pathway

Activation of Nrf2-ARE pathways that leads to expression of ARE-driven genes, including xCT. From (Lee and Johnson, 2004).

Nrf2-ARE has been shown to be necessary for increased cystine uptake through system x_C^- induced by various electrophiles, such as diethyl maleate, arsenite, cadmium and hydroquinone, in BHK21 kidney cells (Sasaki et al., 2002). Upregulating Nrf2 has been shown to increase xCT levels and protect both glial cells and neurons from oxidative stress insults in a phosphatidylinositol 3 kinase (PI3K)/ activate protein kinase B (PKB, aka, Akt) dependent manner (Shih et al., 2003; Wang et al., 2009).

However, knocking out Nrf2 failed to affect MeHg-induced upregulation of xCT, which suggests Nrf2-ARE is not always involved in xCT regulation (Wang et al., 2009).

eIF2-ATF4-AARE pathway

There are two AARE-like sequences in the 5'-flanking region of the xCT gene (Sato et al., 2004; Lewerenz and Maher, 2009). Amino acid response (AAR) is usually triggered by limited uptake of any essential amino acid (Kilberg et al., 2005). It serves as a self-protective effect by globally slowing down protein production and cellular activity by limiting protein synthesis. However, transcription and translation of certain proteins, such as basic leucine zipper and ATF4 is enhanced (Wek et al., 2006). These proteins then regulate gene expression of membrane transporters and growth factors to cope with environmental stresses (Kilberg et al., 2005; Wek et al., 2006; Ameri and Harris, 2008; Kilberg et al., 2009). It is a protective mechanism against a harmful environment, such as changed pH, nutrient levels, and oxidative stress (Duncan and Hershey, 1985; 1987).

eIF2, a heterotrimer composed of eIF2α, eIF2β, and eIF2γ, is an important component of the initiating complex for most of the protein synthesis (Fafournoux et al., 2000). The GDP in eIF2 has to be exchanged for GTP mediated by guanine nucleotide exchange factor eIF2B to successfully form the initiating complex (Matts and London, 1984; Dholakia and Wahba, 1989). Components of eIF2 can be phosphorylated, preventing the attached GDP from being replaced with GTP (Kilberg et al., 2009). Among the three, eIF2α is the most easily phosphorylated (Costa-Mattioli et al., 2007). The two mechanisms of eIF2B inhibition are described below, both of which can lead to increased ATF4 production, and then activation of AARE regulated gene transcription.

When there are not enough available essential amino acids in the cell, the excessive free tRNAs activate the general control nonrepressible protein 2 (GCN2) kinase, which in turn phosphorylates eIF2 (Zhang et al., 2002). Phosphorylated eIF2 has

increased affinity for eIF2B, although it cannot be activated for initiating complex assembly. Therefore, phosphorylated eIF2 becomes a potent competitive inhibitor for eIF2B (Rowlands et al., 1988; Kimball, 1999). Normally, the intracellular eIF2 levels are significantly higher than eIF2B, therefore, phosphorylation of merely 30% of eIF2α is enough to completely block eIF2B activity (Matts and London, 1984; Duncan and Hershey, 1987). Besides GCN2, there are other kinases that are sensitive to other stressors, and become activated leading to eIF2 phosphorylation. These kinases include heme-regulated inhibitor (HRI) activated by heme-deficiency (Han et al., 2001; Lu et al., 2001), double-stranded RNA-activated inhibitor (DAI) or dsRNA-dependent serine/threonine protein kinase R (PKR) activated by viral infection (Hershey, 1989; Proud, 2005) and PKR-like endoplasmic-reticulum (ER)-localized eIF2α kinase (PERK) activated by ER stress (Lu et al., 2004) (Fig. 1.9.). All of these cellular stresses have the potential of activating the same pathways amino acid deprivation activate and inducing system x_C^- expression, but this theory has not yet been tested.

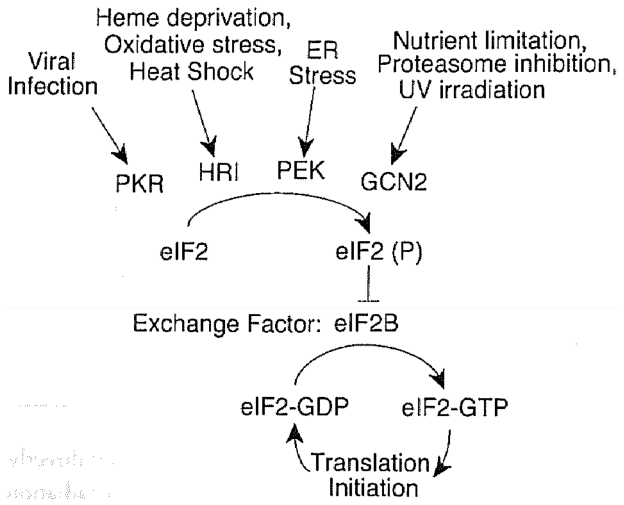

FIGURE 1.9. Regulation of translation initiation by eIF2 phosphorylation. dsRNA-dependent serine/threonine protein kinase R (PKR), heme-regulated inhibitor (HRI), PKR-like endoplasmic-reticulum (ER)-localized eIF2 α kinase (PERK), general control nonrepressible protein 2 (GCN2) are activated in response to various environmental stresses, which leads to phosphorylation of eIF2. Phosphorylated eIF2 inhibits eIF2B mediated guanine nucleotide exchange, in turn, globally slows down translation initiation, but increases ATF4 levels. From (Wek et al., 2006)

Phosphorylation of eIF2B is another mechanism that leads to activation of AARE regulated gene transcription (Proud, 2005) (Fig. 1.10). Glycogen synthetase 3β (GSK3β), a constitutively active kinase, can phosphorylate eIF2B, leading to the loss of its GEF property (Welsh and Proud, 1993) and prevents assembly of the initiation complex. Hormones, mitogens, and growth factors can activate phosphatidylinositol 3(PI3)-kinase (Welsh et al., 1998) and MEK/ERK (Kleijn and Proud, 2000; Quevedo et al., 2000), which can lead to phosphorylation of GSK3β to inactivate it, which removes the GSK3β inhibition effect on eIF2B.

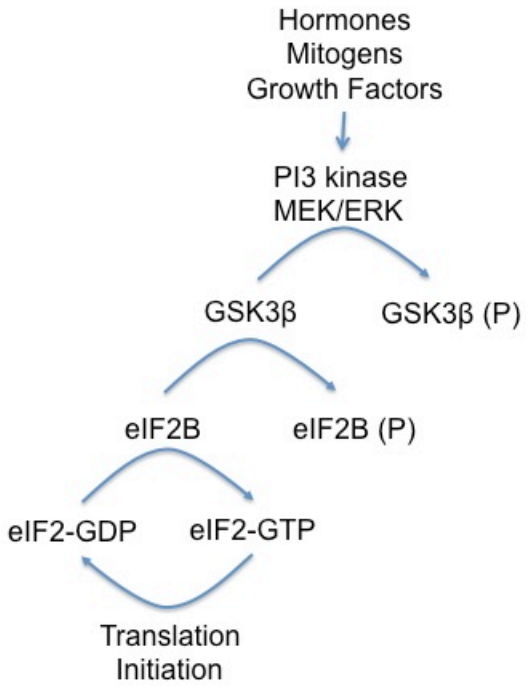

FIGURE 1.10. Regulation of translation initiation by eIF2B phosphorylation. Hormones, mitogens, and growth factors can phosphorylate glycogen synthetase 3β (GSK3β) through phosphatidylinositol 3(PI3)-kinase and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways. Phosphorylation of GSK3β removes the inhibition of eIF2B, and ensures translation initiation.

Phosphorylation of either eIF2 or phosphorylation of eIF2B can lead to global shut down of protein translation, however, there is increased ATF4 production, in turn, activates expression of AARE regulated genes. Mutation studies showed that AARE like sequences in the 5'-flanking region of xCT are essential in both basal and amino acid deprivation induced increase in system x_C^- activity (Sato et al., 2004). Deprivation of cystine, an essential amino acid, activates the ATF4-AARE pathway, and induces an increase in cystine uptake in cultured human fibroblasts after 24 hours (Bannai, 1984). This suggests an involvement of ATF4-AARE in system x_C^- regulation. Further studies showed that 4F2hc is consistently induced under any amino acid deprivation, while xCT is only induced with certain amino acids missing (Sato et al., 2004). This suggests that

the two subunits of system x_C^- have different regulatory mechanisms. Decreasing intracellular eIF2α levels increases HT22 cells resistance to oxidative glutamate toxicity, while increasing intracellular eIF2α can render HT22 cells highly sensitive to glutamate toxicity by decreasing system x_C^- mediated cystine uptake, depleting GSH, and increasing ROS (Tan et al., 2001; Lewerenz and Maher, 2009). This effect is mediated through ATF4 binding to AARE (Lewerenz and Maher, 2009). Therefore, it appears that the ATF4-AARE pathway is an important regulatory mechanism for system x_C^-.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)

NF-κB may be another activator of xCT expression because there is a NF-κB binding site in the 5'-flanking region of xCT (Sato et al., 2001). NF-κB has long been known to play a role in rapid response to calcium influx and harmful cellular stimuli, especially in the immune response (Meffert et al., 2003). LPS is known to activate NF-κB (Sen and Smale, 2010). In mouse peritoneal macrophages, both xCT and 4F2hc mRNA increased in a time dependent manner within 12 hours of LPS treatment (Sato et al., 2001). However, this LPS induced increase in xCT levels is not likely to be mediated through NF-κB because there was no increased nuclear NF-κB caused by the LPS treatment (Sato et al., 2001). Since LPS still induces the activity of system x_C^- in macrophages prepared from Nrf2-deficient mice, it is not likely that an LPS induced increase in system x_C^- activity is mediated through the Nrf2-ARE pathway (Sato et al., 2001). Therefore, despite an NF-κB binding site in the 5'-flanking region of xCT, there is no direct evidence of its role in system x_C^- regulation. The mechanism of LPS inducing increased system x_C^- activity remains unclear.

Activator protein 1 (AP-1)

There are several putative AP-1 binding sites in the 5'-flanking region of xCT, one of them overlaps with the ARE sequence that is essential for response to electrophile reagent activated xCT transcription and translation (Sato et al., 2001). AP-1 transcription factor mediates gene regulation in response to cytokines, growth factors, stress, and infections (Hess et al., 2004). However, its role in xCT regulation has not yet been studied.

Hypoxia inducible factor (HIF)

Hypoxic preconditioning, the protection against a severe hypoxic insult by an earlier mild hypoxic insult, increases xCT levels both transcriptionally and translationally in hippocampus in vivo and in mouse neuronal stem cells (Ogunrinu and Sontheimer, 2010; Sims et al., 2012). This is mostly mediated through HIF-1α, but since siHIF-1α does not completely abolish xCT upregulation in B104 mouse neuronal stem cell cultures after hypoxic preconditioning, the possibility of other intracellular pathways being involved cannot be ruled out (Sims et al., 2012). Also, hypoxia did not induce system x_C^- function in mouse macrophage cultures (Sato et al., 2001). Determining the mechanism of system x_C^- regulation by preconditioning, and when it occurs, requires further study.

cAMP

In rat striatal punches, system x_C^- activity is decreased by 15 minutes of mGluR2/3 agonist treatment, and this effect is mimicked by inhibiting cAMP (Baker et

al., 2002). Also, a two-fold increase in xCT mRNA levels in rat cortical astrocytes was observed after a 10-day incubation with N(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate (dbcAMP), a cAMP analog (Gochenauer and Robinson, 2001). One week dbcAMP treatment potentiates buthionine sulfoximine induced increase in system x_C^- activity in rat primary astrocytes (Seib et al., 2011). There are two consensus PKA phosphorylation sites on human xCT (Baker et al., 2002). However, the exact intracellular pathway by which cAMP is involved in system x_C^- regulation is yet to be investigated.

Growth factors

Our lab was the first to report growth factor effects on system x_C^- function. Insulin-like growth factor 1 (IGF-1) and transforming growth factor-β (TGF-β) can upregulate system x_C^- function in dental pulp cells (Pauly et al., 2011). We also showed that fibroblast growth factor-2 (FGF-2) upregulates system x_C^- function selectively in primary cortical astrocytes (Liu et al., 2011).

FGF-2 RECEPTORS AND INTRACELLULAR SIGNALING PATHWAYS

FGF-2 was the first member isolated and cloned among the FGF family of growth factors in the 1980s. After decades of research, we now know FGF-2 is involved in many nervous system functions. During embryonic development, FGF-2 plays an important role in regulating proliferation, differentiation, and migration; while in adult, FGF-2 plays a critical role in neuronal death, neurogenesis, learning and memory, and lesion repair (Reuss and Bohlen und Halbach, 2003; Eswarakumar et al., 2005). FGF-2 expression is

found in both neuronal and glial cells, with glial cells as its main source (Eckenstein et al., 1991a; 1991b).

FGF-2 can activate all members of the FGF receptor (FGFR) family, with FGFR1 and FGFR2 the prominent forms present in the cerebral cortex (Reuss and Bohlen und Halbach, 2003). Like any typical tyrosine kinase receptor, FGFRs are composed of an extracellular ligand-binding domain composed of three immunoglobulin-like domains, a single transmembrane domain, and an intracellular domain with catalytic protein tyrosine kinase activity (Mohammadi et al., 1996a). Two molecules of FGF-2 bind to FGFRs, which triggers dimerization and activation of tyrosine kinase activity through autophosphorylation (Schlessinger et al., 1995; Mohammadi et al., 1996b). Previous studies from our lab showed that in primary mouse cortical culture, FGFR1 is the most prevalent receptor, followed by FGFR2, and both are mostly expressed in astrocytes (Fig. 1.11) .

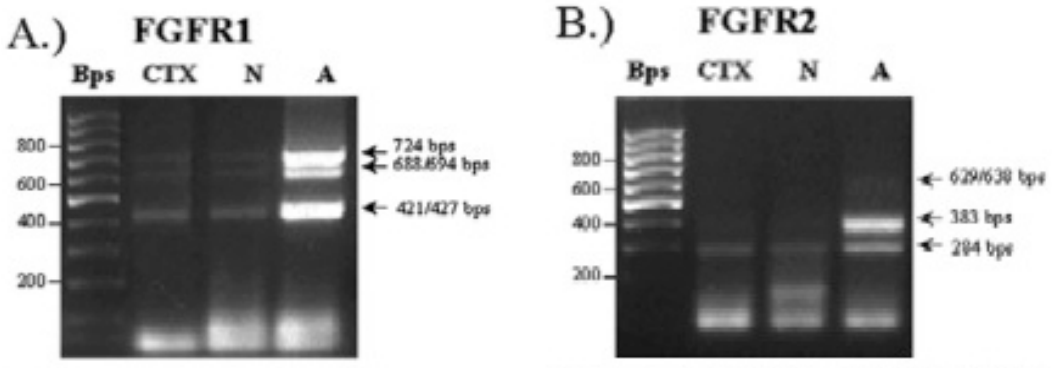

FIGURE 1.11. Expression of mRNA for FGFR1 and FGFR2 in cerebral cortex, neuronal-enriched cultures, and glial-enriched cultures.

Bps, base pairs; CTX, cerebral cortex; A, glia-enriched cortical cultures; N, neuronal-enriched cortical cultures. Three isoforms of both FGFR1 and FGFR2 were detected. From (Lobner and Ali, 2002).

Upon activation, at the carboxy terminal tail of the FGFR, autophosphorylation on a tyrosine residue (Tyr766) creates a specific binding site for PLC (Mohammadi et al., 1992; 1996a) and activates it to catalyze the hydrolysis of PIP_2 to generate two secondary messengers: IP_3 and DAG. Also, fibroblast growth factor receptor substrate 2 (FRS2) constitutively docks at the juxtamembrane domain of FGFRs (Reuss and Bohlen und Halbach, 2003; Eswarakumar et al., 2005). In response to FGFR activation, the docking proteins become tyrosine phosphorylated and recruit protein complexes and activate multiple intracellular signal transduction pathways, such as PI3K/Akt and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways (Kouhara et al., 1997; Ojeda et al., 2011). The PLC activation mediated effects are independent from the FRS2 mediated effects (Fig. 1.12.).

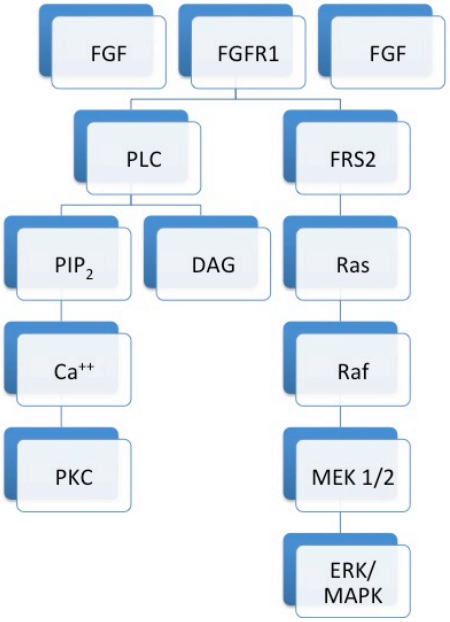

FIGURE 1.12. FGF-FGFR1 intracellular signaling pathways.

Activation of fibroblast growth factor receptor 1 (FGFR1) requires binding of two fibroblast growth factor-2 (FGF-2) molecules, and two independent intracellular pathways are activated: phospholipase C (PLC), which leads to generation of two secondary messengers, inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG); fibroblast growth factor receptor substrate 2 (FRS2) mediated activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway.

FGF-2 regulation, function and dysfunction in diseases

FGF-2 in ischemia/stroke

FGF-2 expression is upregulated rapidly in the brain after lesioning or ischemic insult (Frautschy et al., 1991). Most of the FGF-2 expression is in astrocytes, with only some of the neurons close to the lesion core showing increased FGF-2 levels (Wei et al., 2000). Because of the success of FGF-2 in animal models of ischemia, it has been considered as a candidate for stroke treatment in humans (Ay et al., 1999; Ren and Finklestein, 2005). However, clinical trials of trafermin (recombinant human FGF-2) administration in human acute ischemic stroke were unsuccessful. Administration of

FGF-2 failed to produce any significant protection in the treated groups as compared with the control groups (Clark et al., 2000; Bogousslavsky et al., 2002). Instead, there was an increased mortality rate in the treated patients (Clark et al., 2000; Bogousslavsky et al., 2002). One hypothesis was that although FGF-2 is a cationic peptide that can be transported across the blood brain barrier at a higher rate than most other growth factors (Deguchi et al., 2000), due to the limited transportation efficiency, high doses were required to produce the central effect, which caused more peripheral side effects, such as vasodilatation (Cuevas et al., 1991; Rosenblatt et al., 1994). In light of our finding of FGF-2 induced increased system x_C^- function, enhanced excitotoxicity must also be considered as a possibility.

FGF2 in major depression/anxiety

FGF-2 was recently suggested as a potential antidepressant (Turner et al., 2008c; Perez et al., 2009). Human postmortem studies indicate decreased FGF system function in the frontal cortex (Evans et al., 2004) and hippocampus (Gaughran et al., 2006) of patients who suffered from major depression. Patients with a history of antidepressant treatment showed attenuated FGF signaling dysregulation (Evans et al., 2004). In rodent studies, both FGF-2 and FGFR1 levels were decreased in hippocampus following social defeat (Turner et al., 2008a). Also, in a rodent model where rats were selectively bred to enhance different emotional behavior, lowered FGF-2 levels in the hippocampus were observed in the high anxiety group comparing to the low anxiety group (Perez et al., 2009). Exposure to an enriched environment effectively reduced anxiety behavior of the high anxiety group, and this was accompanied by increased FGF-2 levels in the

hippocampus (Perez et al., 2009). Chronic exogenous FGF-2 treatment produced the same behavioral effects (Perez et al., 2009). Furthermore, administration of FGF-2 during development prevented the low responders from exhibiting high anxiety-like behavior in adult (Turner et al., 2011). Administration of FGF-2 was also reported to reduce anxiety-like behaviors in the forced swim test model (Turner et al., 2008c). Antidepressants were shown to be able to increase FGF-2 levels in brain regions, such as prefrontal cortex, hippocampus, and striatum in rodents (Mallei et al., 2002; Maragnoli et al., 2004). Therefore, the evidence of FGF-2 dysregulation in depression is strong.

FGF-2 and schizophrenia

Schizophrenia is believed to be a result of interplay of both genetic predisposition and environmental risk factors (Tsuang, 2000). Prenatal stress has been suggested as an important environmental risk factor (Tsuang, 2000; Terwisscha van Scheltinga et al., 2010), and it has been shown to decrease FGF2 expression in rat entorhinal cortex and striatum, as well as FGF-2 levels in response to acute and chronic stresses in various brain regions in adult (Fumagalli et al., 2005). Social defeat is another risk factor for schizophrenia (Selten and Cantor-Graae, 2005; 2007). In adult rats, both FGF-2 and FGFR1 mRNA in hippocampus are downregulated after social defeat (Turner et al., 2008a). Smoking of cannabis is another environmental factor that is known to increase the risk of developing schizophrenia (Moore et al., 2007). Cannabinoid receptor type 1 antagonists have been shown to inhibit axonal growth responses stimulated by FGF-2 (Williams et al., 2003). Decreased FGFR1 mRNA levels in dentate gyrus were reported in a mouse knockout model of neuronal PAS domain protein 3, a familial mutation

involved in schizophrenia (Kamnasaran et al., 2003; Pieper et al., 2005), and two different FGFR1 knockout mice showed "schizophrenia-like characteristics" (Ohkubo et al., 2004; Shin et al., 2004; Klejbor et al., 2006).

Schizophrenia is also believed to involve impairments in connections between neurons, and between different brain regions, which could result from abnormal development (Foucher and Luck, 2006). FGF-2 has been shown to promote dendritic growth in the hippocampus (Rai et al., 2007), as well as promote embryogenesis and increase cerebral cortex volume (Vaccarino et al., 1999). FGF-2 knockout mice show decreased neuronal number in the cerebral cortex (Turner et al., 2006).

Several antipsychotic drugs have been shown to change FGF-2 mRNA levels in both human (Hashimoto et al., 2003) and rat hippocampus (Gómez-Pinilla et al., 2000; Ovalle et al., 2001; Fumagalli et al., 2004; Maragnoli et al., 2004). On the other hand, postmortem studies showed that FGF-2 mRNA in the hippocampus was decreased in clozapine treated schizophrenic patients compared with the nontreated (Gaughran et al., 2006).

Therefore, there is a strong association between FGF-2 dysregulation and schizophrenia. Schizophrenia is also associated with decreased glutathione and glutamate dysfunction in the brain (Pérez-Neri et al., 2006; Ballatori et al., 2009). These suggest the possibility of FGF-2 dysregulation mediated effects being due to its regulation of system x_C^-.

FGF-2 and addiction

FGF-2 has also been suggested to be involved in addiction. Agonists of dopaminergic D2 receptors increase FGF-2 gene expression in rat prefrontal cortex and hippocampus (Fumagalli et al., 2003). Both acute and chronic exposure to cocaine increases FGF-2 expression in rat striatum, prefrontal cortex, hippocampus, and frontal cortex (Fumagalli et al., 2006). Rats that were selectively bred for enhanced drug seeking behavior have higher FGF-2 expression levels (Turner et al., 2009). Postnatal administration of FGF-2 enhances acquisition of cocaine self-administration in adults (Turner et al., 2009). However, the functional involvement of FGF-2 in addiction has been little studied.

SYSTEM XC- CONTRIBUTION TO CELL DEATH/SURVIVAL

Oxidative glutamate toxicity

In vitro experiments demonstrated that immature neurons rely heavily on system x_C^- activity to take up cystine (Murphy et al., 1990). They lack glutamatergic receptors; so instead of causing excitotoxicity, high levels of extracellular glutamate cause toxicity in these cells by competitively inhibiting system x_C^-, causing oxidative glutamate toxicity (Murphy and Baraban, 1990; Schubert and Piasecki, 2001; Lewerenz et al., 2006). The oxidative glutamate toxicity is characterized by depletion of GSH (Miyamoto et al., 1989; Murphy et al., 1989; Murphy and Baraban, 1990; Murphy et al., 1990), and has characteristics of both apoptosis and necrosis (Tan et al., 1998). Similar oxidative glutamate toxicity is also observed in neuroblastoma-primary retina hybrid cells (N18-

RE105) (Miyamoto et al., 1989), primary rat hippocampal neurons (Murphy et al., 1989), a mouse hippocampal cell line (HT-22) (Tan et al., 2001), and primary oligodendrocytes (Oka et al., 1993).

Increased system x_C^- activity: protection against oxidative stress

Upregulation of system x_C^- has been shown to be protective against oxidative stress in multiple systems. Fast proliferating primary brain glial-derived tumor cells produce high levels of ROS. These cells rely primarily on system x_C^- to take up cystine to produce GSH, therefore, inhibiting system x_C^- decreases the growth of these tumor cells (Chung et al., 2005). High system x_C^- activity might contribute to the high tolerance of human ovarian cancer cells to anticancer treatments (Okuno et al., 2003). In mouse neural stem cells, hypoxic preconditioning increases system x_C^- function and strengthens the cell's resistance to oxidative glutamate toxicity (Sims et al., 2012). This preconditioning produced protection is absent when either system x_C^- is blocked by S-4-carboxyphenyl glycine (CPG), or GSH synthesis is blocked by buthionine sulphoximine (Sims et al., 2012). IGF-1 and TGF-β increase cystine uptake through system x_C^- on dental pulp cells, which leads to protection against oxidative stress (Pauly et al., 2011). Retinoic acid increases SH-SY5Y cell (a human dopaminergic neuroblastoma cell line) tolerance for 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium ion (MPP+) compared to untreated cells (Crockett et al., 2011). This is because retinoic acid increases system x_C^- activity, which increases GSH levels and protects the cells from these oxidative insults (Crockett et al., 2011). Upregulating Nrf2, one of the well-characterized pathways that regulate xCT expression, is also known to protect both glial

cells and neurons from oxidative stress insults in a PI3K/Akt dependent manner (Shih et al., 2003; Wang et al., 2009). Ceftriaxone protects fibroblasts and the hippocampal cell line HT22 from oxidative glutamate toxicity by upregulating system x_C^- through the Nrf2-ARE pathway (Lewerenz et al., 2009). All of this data suggests that increasing system x_C^- can increase cystine uptake, which can lead to increased glutathione production, and provide protection against oxidative stress.

Increased system x_C^- activity: enhanced excitotoxicity

Increasing system x_C^- activity is not always protective. Activated microglia have increased glutamate release via system x_C^-, which causes oligodendrocyte excitotoxicity through AMPA receptors (Domercq et al., 2007). Interleukin 1β (IL-1β) upregulates system x_C^- activity selectively on astrocytes, but not neurons (Jackman et al., 2010). This increased system x_C^- activity is responsible for exacerbating neuronal cell death mediated by excitotoxicity when the cultures are exposed to hypoxia (Fogal et al., 2007) or glucose deprivation (Jackman et al., 2012). These studies demonstrate that when system x_C^- activity is increased, glutamate release is also increased, which can potentially exacerbate excitotoxicity.

POTENTIAL ROLE OF SYSTEM XC- IN NEUROLOGICAL DISORDERS

Alzheimer's disease (AD)

AD is a common neurodegenerative disorder in elderly people. It is characterized by progressive memory loss and impaired cognitive function. Amyloid-β (Aβ) plaques and formation of neurofibrillary tangles are the characteristic hallmarks of this disease.

Aβ is a normal product of the brain, therefore, the abnormal deposition must be due to an over production and/or impaired clearance. Astrocytes can degrade Aβ (Koistinaho et al., 2004; Pihlaja et al., 2011). However, Aβ can make astrocytes reactive, which can contribute to neuronal degeneration (Olabarria et al., 2010). There are increased levels of phosphorylated eIF2α (Chang et al., 2002; Unterberger et al., 2006) and ATF4 (Lewerenz and Maher, 2009) in AD patient's brains, which are shown to be involved in xCT regulation (Lewerenz and Maher, 2009). In fact, in PC12 cells, induction of eIF2α phosphorylation using the specific eIF2α phosphatase inhibitor, salubrinal, increased ATF4 levels, system x_C^- activity, and resistance to Aβ toxicity (Lewerenz and Maher, 2009). Therefore, it is most likely that the increase of eIF2α and ATF4 is an adaptation to the increased Aβ accumulation. However, secreted Aβ precursor protein (APP) can activate microglia and stimulate system x_C^- mediated glutamate release from microglia, which leads to decreased synaptic density and neuronal viability (Barger and Basile, 2001). Therefore, it is not clear whether upregulation of system x_C^- during AD is beneficial or harmful.

Parkinson's disease (PD)

PD is a common nervous system disorder in the elderly that is characterized by degeneration of dopaminergic cells in the substantia nigra, resulting in symptoms such as difficulties in initiating and finishing voluntary movements, tremors, stiff movements, and eventually leads to muscle atrophy (Obeso et al., 2010). Extensive postmortem studies have reported that increased oxidative stress, decreased GSH, and increased oxidative damage to lipids, protein and DNA are observed in PD (Jenner and Olanow,

1996; Simonian and Coyle, 1996). Increased striatal glutamate (Massie et al., 2011) and decreased nigral glutathione (Schulz et al., 2000) have both been indicated to contribute to dopaminergic cell degeneration.

Involvement of system x_C^- in animal models of PD has been studied. xCT is upregulated in the striatum three weeks after 6-OHDA lesion of the nigrostriatal area (Massie et al., 2008). Also, 24 hour 6-OHDA treatment increases system x_C^- mediated cystine uptake in C6 glioma cells in a dose dependent manner (Massie et al., 2008). In xCT null mice, striatal glutamate levels are significantly lower compared to control suggesting that system x_C^- is responsible for the majority of extracellular glutamate (Baker et al., 2002; De Bundel et al., 2011; Massie et al., 2011). The absence of xCT decreased the susceptibility of substantia nigra dopaminergic neurons to 6-OHDA toxicity (Massie et al., 2011). These data suggest that system x_C^- function may have a net damaging effect in PD, but it must be kept in mind that the 6-OHDA lesion model is not an ideal model of PD.

Amyotrophic lateral sclerosis–Parkinsonism dementia complex (ALS-PDC)

ALS-PDC is a neurological disease that is also known as lytico-bodig to the Chamorro people on the island of Guam (Reed and Brody, 1975). The disease has aspects of ALS, PD, and AD: muscular weakness, spasticity, flaccid paralysis and atrophy, and spinal-cord pathology similar to that are seen in ALS, tremors seen in PD, neurofibrillary tangles similar to these occurring in brain tissue in AD, and dementia that is seen both in PD and AD (Spencer et al., 1987; Liu et al., 2009). The disease has been known to be occurring on Guam for about 200 years (Kurland and Mulder, 1954).

However, an outbreak of ALS-PDC in the 1940s to 1960s gained attention because the incidence in the Chamorro population was 100 times higher than ALS elsewhere (Kurland and Mulder, 1954; Spencer et al., 1986). The etiology of ALS-PDC remains uncertain, dietetic, environmental and genetic factors have all been considered (Ince and Codd, 2005).

At first, genetic factors were considered as the cause since certain villages and families had higher rates of ALS-PDC. However, no genetic mutation that was found that could account for the disease, and later studies indicated that adopting the local life style and traditional diet had a significant association with the disease (Garruto et al., 1981).

β-N-methylamino-L-alanine (BMAA)

BMAA is a non-protein amino acid that is one of the many potential toxins found in cycad plants (Spencer et al., 1987; Cox et al., 2003). It was proposed as a possible environmental trigger for ALS-PDC on Guam (Spencer et al., 1987). The local Chamorro people collect the cycad seeds for food and medicine (Bradley and Mash, 2009). The local flying foxes (fruit bats) also feed on cycad seeds and it was found that BMAA accumulates at high levels in these bats (Cox and Sacks, 2002; Cox et al., 2003; Monson et al., 2003). Flying fox stew was served as a local courtesy dish until its extinction in the 1960s, after which time the incidence of ALS-PDC declined.

BMAA was found to mimic glutamate and activate glutamatergic receptors when bicarbonate is present (Weiss and Choi, 1988; Richter and Mena, 1989) (Fig. 1.13), and BMAA was found to cause excitotoxicity when physiological concentration of

bicarbonate was present in the media (Weiss and Choi, 1988; Weiss et al., 1989b). The BMAA carbonate competes with glutamate at synapses (Richter and Mena, 1989) and was shown to have direct binding to the NMDA receptors (Weiss et al., 1989a).

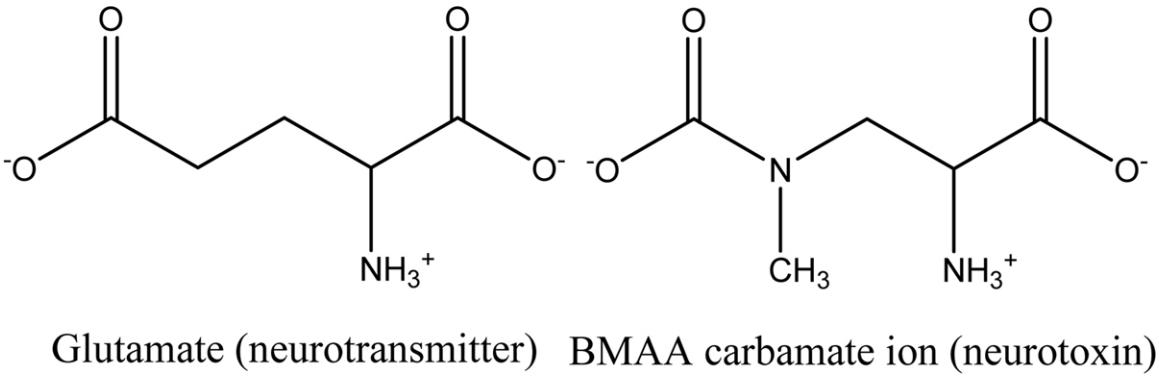

FIGURE 1.13. Structural similarities between the neurotransmitter L-glutamate and the neurotoxic carbonate adduct of BMAA.
From (Erdner et al., 2008)

Oral bioavailability conducted in cynomolgous monkeys indicated that 80% of the administered BMAA was absorbed into the systemic circulation (Duncan et al., 1992). More specifically, BMAA crosses the blood-brain barrier, and is retained at high levels in the central nervous system (Kisby et al., 1988; Duncan et al., 1991; Smith et al., 1992).

BMAA bicarbonate mimicking glutamate is not the only mechanism of BMAA actions, since it also increases calcium levels in dissociated rat brain cells in bicarbonate-free media (Brownson et al., 2002). In previous studies, we found that BMAA can cause cell death of primary cultured cortical neurons by activating glutamatergic receptors such as NMDA and mGluR5 (Lobner et al., 2007). In this thesis we present evidence that BMAA also acts as a substrate for system x_C^- and by doing so both inhibits cystine uptake and stimulates glutamate release (Liu et al., 2009).

Heavy metals

Heavy metal toxicity was another hypothesis for ALS-PDC (Johnson and Atchison, 2009). However, no clear evidence of heavy metal toxicity was discovered. Since the outbreak of Minamata disease in Japan in 1956, due to an industrial release of methylmercury (MeHg) (Harada, 1995), MeHg has received attention for its neurotoxic actions both during development and in adults.

MeHg is an environmental neurotoxin that is most often consumed through contaminated seafood (Rush et al., 2012a). It is not directly associated with ALS-PDC, but has been implicated in neurodegenerative diseases (Wang et al., 2008). In vivo studies suggest that the major localization of MeHg is in glial cells (Charleston et al., 1995). Further studies showed that in glial cells MeHg decreases GSH levels (Aschner et al., 1994) and disrupts glutamatergic and GABAergic homeostasis (Schousboe et al., 1992). In astrocytes, MeHg exposure activates the Nrf2-ARE pathway to upregulate a series of antioxidative proteins and increases GSH levels (Wang et al., 2008; Niciu et al., 2012). We have shown that MeHg induces increased system x_C^- activity in primary mixed neuronal and glial cortical culture (Fonnum, 1984; Rush et al., 2012b). Interestingly, we also showed that MeHg and BMAA synergistically deplete cellular GSH levels and induced toxicity to neuronal cells in primary cultures (Petroff, 2002; Schousboe and Waagepetersen, 2007; Rush et al., 2012a).

Ischemia/Stroke

Stroke is caused by disturbance in the blood supply to the brain, which leads to rapid loss of brain functions and often results in permanent neurological damage (Meister

and Anderson, 1983; Dringen et al., 2000; Kalra and Ratan, 2008). It is one of the leading causes for death and disability, especially in the elderly population (Erecińska and Silver, 1990; Choi, 1998). Injury induced increases in IL-1β from microglia may exacerbate ischemic injury, because IL-1 receptor type I deficient mice show smaller infarcts compared to wild type animals following 90 minute reversible middle cerebral artery occlusion (Dingledine et al., 1999; Fogal et al., 2007). In vitro data shows that IL-1β selectively upregulates system x_C^- levels on astrocytes but not neurons, and that glutamate released from system x_C^- enhances hypoxic neuronal death (Jackman et al., 2010; Niciu et al., 2012). Therefore, IL-1β mediated ischemic damage may be mediated by its upregulation of system x_C^-. In contrast, erythropoietin has been shown to be protective against ischemic neuronal injury (Noguchi et al., 2007; Niciu et al., 2012). Interestingly, erythropoietin increases xCT expression in B104 cells, and this upregulation can protect the cells from oxidative glutamate toxicity (Dingledine et al., 1999; Sims et al., 2010). Ischemic preconditioning, which protects cells from further ischemic stress, has also been shown to upregulate system x_C^- (Jonas et al., 1994; Sims et al., 2012). It is possible that the net effect of upregulation of system x_C^- on ischemia induced cell death is dependent on the cell type on which it is upregulated.

Glioma

Gliomas are a type of tumor that arise from glial cells and are the most common type of brain tumor (Sontheimer, 2003; Clem and Barth, 2006). Glioma cells have increased need for GSH because of their fast dividing rate. When glioma cells experience oxidative stress, system x_C^- is rapidly upregulated to maintain the GSH levels

(Schneggenburger et al., 1993; Kim et al., 2001). Glioma cells release a large amounts of glutamate into the extracellular space because of the high system x_C^- activity (Dingledine et al., 1999; Ye and Sontheimer, 1999), but they also lack sodium-dependent glutamate transporters that clear glutamate from extracellular space (Dingledine et al., 1999; Ye et al., 1999). The combination of these actions cause excitotoxicity to surrounding neuronal cells and promotes glioma growth at the same time (Béhé et al., 1995; Premkumar and Auerbach, 1997; Takano et al., 2001). Therefore, glioma cells utilize system x_C^- to kill surrounding neuronal cells by releasing glutamate to cause excitotoxicity to make room for additional growth, and take up sufficient amounts of cystine through system x_C^- to make GSH to reduce oxidative stress (Patneau and Mayer, 1990; Burnashev et al., 1995; Sontheimer, 2003; 2008). The high system x_C^- activity also makes the tumor cells more resistant to drugs (Nowak et al., 1984; Lo et al., 2008). In mouse models of glioma tumors, the increased glutamate release can cause epileptic activity, which is a common symptom in the majority of glioma patients (Nowak et al., 1984; Dingledine et al., 1999; Buckingham et al., 2011). Because inhibiting system x_C^- suppresses glioma growth by depleting GSH (Johnson and Ascher, 1987; Schell et al., 1995; Chung and Sontheimer, 2009; Guan et al., 2009), it has been suggested as a treatment for gliomas (Chung and Sontheimer, 2009; Grienberger and Konnerth, 2012).

Addiction

According to the American Society of Addiction Medicine, "addiction is a primary, chronic disease of brain reward, motivation, memory and related circuitry." Decreased levels of basal extracellular glutamate resulting from disrupted system x_C^-

function following chronic cocaine administration has been implicated in the pathophysiology of drug addiction (Conn and Pin, 1997; Baker et al., 2003; Ferraguti and Shigemoto, 2006; Kim et al., 2008; Lewerenz et al., 2012). System x_C- provides most of the extrasynaptic glutamate in nucleus accumbens, which can activate group II mGluRs that are autoreceptors localized on presynaptic terminals (Baker et al., 2002; Niciu et al., 2012). Intracellularly, group II mGluRs couple to $G_{i/o}$ to cause decreased cAMP levels and eventually lead to decreased neurotransmitter release from the presynaptic terminal (Kilbride et al., 2001; Niciu et al., 2012). Decreased group II mGluRs function has been reported after chronic cocaine administration (Xi et al., 2002; Niciu et al., 2012). Interestingly, before reinstatement of cocaine seeking, infusing cystine into the nucleus accumbens of chronic cocaine administrated animals not only eliminated the decreased extracellular glutamate levels, but also prevented acute cocaine induced increase in synaptic glutamate release (Choi, 1987; Baker et al., 2003). Administration of N-acetylcysteine, a cysteine pro drug, prevented cocaine induced reinstatement (Bains and Shaw, 1997; Choi, 1998; Baker et al., 2003; Moussawi et al., 2009). A logical explanation for these results is that chronic cocaine administration decreases glutamate release from system x_C-, as well as decreases presynaptic group II mGluRs functions, which leads to decreased presynaptic inhibition of neurotransmitter release. After extinction, following acute cocaine administration, increased neurotransmitter release elicits reinstatement. However, when extrasynaptic glutamate levels are restored through increasing glutamate release by driving system x_C- with cystine, presynaptic inhibition is restored, which prevents excessive synaptic glutamate release.

The exact mechanism by which cocaine decreases system x_C- activity is unknown. However, injections of N-acetylcysteine before each self-administration prevented chronic cocaine intake escalation (McDonald et al., 1998; Madayag et al., 2007), as well as blunted reinstatement (Pitt et al., 2000; Smith et al., 2000; Madayag et al., 2007; Kau et al., 2008). These studies showed that promotion of system x_C- function can prevent cocaine induced synaptic plasticity to a certain extent. The beneficial effect of N-acetylcysteine does not seem to be limited to preventing the synaptic plasticity caused by cocaine administration, but also involves the ability to reverse some plasticity caused by cocaine administration. Infusion of N-acetylcysteine after drug administration also restores basal extracellular glutamate levels (Yoshioka et al., 1996; Bannerman et al., 2007; Moussawi et al., 2009), and blunts reinstatement (Erecińska and Silver, 1990; Amen et al., 2011). Ceftriaxone, which induces xCT expression and increases system x_C- function (Barbour et al., 1988; Zerangue and Kavanaugh, 1996a; Lewerenz et al., 2009), was also shown to be able to restore cocaine administration induced decrease in extracellular glutamate back to normal and prevent relapse to cocaine seeking (Ventura and Harris, 1999; Knackstedt et al., 2010; Witcher et al., 2010).

GENERAL METHODS

In this dissertation all of the studies utilize primary cell cultures. Following is an overview of the essential methods used in the studies, with a brief assessment of the advantages and limitations of these procedures.

Cortical Culture

Cortical cell culture is a well-established system for modelling aspects of in vivo systems. For these studies, the cortex is dissected from mice on gestational day 15. Cortexes from the same litter are dissociated, then trypsinized before plating in Eagle's minimum essential media containing 5% heat-inactivated horse serum and 5% fetal bovine serum on poly-D-lysine and laminin coated 24 well plates. Cultures are then incubated in a 5% CO_2 incubator at 37°C for two weeks before being used for experiments. Every six to eight embryos yield one plate. In a mature mixed plate the neurons (~200,000) extend out axons and dendrites to form complicated networks, below the neurons, glial cells (~100,000) form a confluent layer. Similarly, we can also make neuronal-enriched and glia-enriched cultures. By adding cytosine arabinocide, a mitotic inhibitor, 2-3 days after plating, glial replication is eliminated producing neuronal-enriched cultures. By delaying the dissection until postnatal day one, neurons do not survive producing glia-enriched cultures. These procedures provide a complex, but easily reproducible, in vitro system in which to perform studies.

However, there are some limitations of this culture system. The cells can only be maintained in vitro for a maximum of about 3 weeks, and even during that time cells are continually dying, this limits the ability to mimic low-dose, long-term insults. Most of our experiments are performed on cultures DIV 13-15, with insults lasting 24-48 hours. Also, as a common limitation of all cell culture studies, the cells are maintained in a media that is optimized for cell health, and is not exactly the same as that in vivo. These limitations should always be kept in mind when interpreting cell culture studies.

Cell death assay

To quantify the cell death/survival, plate reader based assays are used. Lactate dehydrogenase (LDH) is a large cytosolic enzyme that is normally retained in the cells. However, in the event of cell damage/death, the cell membrane is compromised and the cellular contents, including LDH, leak out into the extracellular culture media. The amount of LDH being released is proportional to the amount of dead cells. The assay for LDH involves the fact that it catalyzes the conversion of pyruvate to lactic acid using NADH. By sampling the extracellular media, then adding pyruvate and NADH, the amount of LDH present can be determined by measuring the rate of NADH loss using an absorbance based plate reader. By comparing experimental groups to the control group and full kill (neuronal full kill is achieved by adding 500 μM NMDA to induce excitotoxicity; glial full kill is achieved by adding 20 μM A23187, a calcium ionophore, to cause calcium overload to induce cell death), the relative percentage of cell death can be obtained. Another assay we use to measure neuronal injury is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT can be reduced by mitochondrial reductase enzymes forming a dark purple formazan compound. By measuring the formazan levels, an indirect measure of cell viability is obtained. The results of these automated assays are confirmed for key experiments by trypan blue staining, which is a blue dye that only stains the cells whose membrane is compromised.

The automated assays provide a fast and accurate way of assessing cell death. However, one should keep in mind that reagents that can potentially alter the function of the enzymes that are used for the assays may alter the results. For example, LDH function can be altered by hypoxia (Soñanez-Organis et al., 2012), anoxia (Xiong and

Storey, 2012), and growth factors (Matrisian et al., 1985). Also, these assays utilize changes in absorbance to detect color changes to measure enzyme activity. Therefore, reagents that interfere with absorbance detection may dramatically change the results. Because of these confounding factors visual examination and estimation of cell death is always performed.

^{14}C-cystine uptake

Under physiological conditions, system x_C- transports cystine into cells in exchange for glutamate being transported out. To assess system x_C- activity, we take advantage of the amino acid transport function of system x_C- and measure the uptake of ^{14}C-cystine. The advantage of measuring cystine uptake, as opposed to glutamate uptake or release, is that, in our primary cortical cultures, cystine does not directly interact with receptors on the cell membrane. In contrast, there are a large amount of glutamatergic receptors and transporters present on the membrane. However, there are some drawbacks to using ^{14}C-cystine uptake as measure of system xc- function. Cystine taken up by cells is quickly broken down into cysteine, and this can happen extracellularly as well. Therefore, the uptake data may be a mixture of cystine/cysteine uptake. Since system x_C- only transports cystine, not cysteine, this problem can be addressed by blocking system x_C- to dissect out the system x_C- mediated uptake activity. Intracellularly, cysteine can be used to synthesize glutathione, and other cysteine containing molecules, which can be exported. Therefore, with long-term treatment times the uptake data may be a compromised by changes in the efflux rate. To address this concern we compared ^{14}C-cystine uptake for 2 and 20 minutes with or without 24 hour FGF-2 treatment (Fig. 1.14).

The two time points gave similar results, suggesting that 20 minutes is not too long of a period to measure uptake under these conditions.

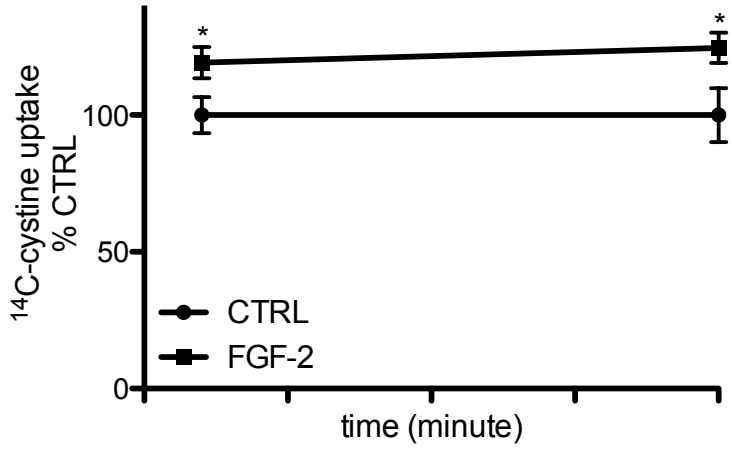

FIGURE 1.14. 24 hour FGF-2 treatment on 2 min and 20 min cystine uptake

Following 24 hour exposure to FGF-2 (100 ng/ml), ^{14}C-cystine uptake was measured for 2 min and 20 min in astrocyte-enriched cultures. Data are expressed as ^{14}C-cystine uptake in CPM per microgram protein normalized to control (mean±s.e.m., n=4) (A). * indicates significantly different from control uptake.

CHAPTER II

β-N-methylamino-L-alanine induces oxidative stress and glutamate release through action on system x_C^-

ABSTRACT

β-N-methylamino-L-alanine (BMAA) is a non-protein amino acid implicated in the neurodegenerative disease amyotrophic lateral sclerosis and Parkinson-dementia complex (ALS-PDC) on Guam. BMAA has recently been discovered in the brains of Alzheimer's and ALS patients in North America and is produced by various species of cyanobacteria around the world. These findings suggest the possibility that BMAA may be of concern not only for specific groups of Pacific Islanders, but for a much larger population. Previous studies have indicated that BMAA can act as an excitotoxin by acting on the NMDA receptor. We have shown that the mechanism of neurotoxicity is actually three-fold; it involves not only direct action on the NMDA receptor, but also activation of metabotropic glutamate receptor 5 (mGluR5) and induction of oxidative stress. We now explore the mechanism by which BMAA activates the mGluR5 receptor and induces oxidative stress. We found that BMAA inhibits the cystine/glutamate antiporter (system x_C^-) mediated cystine uptake, which in turn leads to glutathione depletion and increased oxidative stress. BMAA also appears to drive glutamate release via system x_C^- and this glutamate induces toxicity through activation of the mGluR5 receptor. Therefore, the oxidative stress and mGluR5 activation induced by BMAA are both mediated through action at system x_C^-. The multiple mechanisms of BMAA toxicity, particularly the depletion of glutathione and enhanced oxidative stress, may account for its ability to induce complex neurodegenerative diseases.

INTRODUCTION

In the 1950s it was observed that a substantial number of Chamorros, the native people of Guam, began developing a disease that showed combined symptoms of amyotrophic lateral sclerosis and Parkinson's dementia complex (ALS-PDC). The idea that BMAA may be involved in this disease began in the 1980s when it was found that BMAA was present in cycad seeds which were consumed by the Chamorros (Nunn et al., 1987; Amara, 1992; Kanai and Hediger, 1992; Storck et al., 1992; Rothstein et al., 1994; 1996; Diamond and Jahr, 1997; Lehre and Danbolt, 1998) and that injection of BMAA into monkey brains induced a Parkinson-like disease (Spencer et al., 1987; Sibson et al., 1998). Since its initial proposal, the BMAA hypothesis for the development of ALS-PDC on Guam has been controversial (Rothstein et al., 1996; Asztely et al., 1997; Rusakov and Kullmann, 1998; Cox and Sacks, 2002; Papapetropoulos, 2007). It was challenged by findings that the levels of BMAA in cycad seeds are too low to cause damage to the brain or the spinal cord, particularly because the Chamorros thoroughly wash the cycad seeds, leading to very low levels of BMAA being consumed (Duncan et al., 1990; Hardingham et al., 2002; Riccio and Ginty, 2002; Ivanov et al., 2006; Léveillé et al., 2008; Xu et al., 2009; Hardingham and Bading, 2010). The BMAA hypothesis was largely abandoned until the last few years.

A number of recent studies have brought the BMAA hypothesis back into prominence. First, it was shown that BMAA is biomagnified. BMAA is produced by cyanobacteria that live on cycad plants; it accumulates in the cycad seeds, which are eaten by fruit bats, which are in turn eaten by the Chamorros (Cox et al., 2003; Lipton and Chen, 2004; Xia et al., 2010). This system provides a new mechanism by which

BMAA can be accumulated to high levels in humans. Second, it was shown that BMAA could become protein-associated. This property allows for BMAA to build up in tissue and provides a mechanism for slow release (Lipton and Chen, 2004; Murch et al., 2004b; Chen and Lipton, 2006; Xia et al., 2010). This slow release may provide a possible explanation for the delayed onset of ALS-PDC following the time of BMAA consumption (Ince and Codd, 2005; Niciu et al., 2012). Third, cyanobacteria present throughout the world have been shown to produce BMAA (Futamachi and Pedley, 1976; Roitbak and Fanardjian, 1981; Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008; Johnson et al., 2008; Metcalf et al., 2008). Also, BMAA was found not only in brain samples of ALS-PDC patients from Guam, but also in the brains of ALS and Alzheimer's disease patients from North America, but not in patients who died of other causes (Venance et al., 1997; Murch et al., 2004a; Pablo et al., 2009). These results suggest that BMAA may be of concern not only for people on select Pacific islands, but for a much larger population. Fourth, BMAA at lower concentrations than previously believed is neurotoxic. The original studies in cortical cell culture found that very high BMAA concentrations (1-3 mM) were required to induce neuronal death (Ross et al., 1987; Weiss and Choi, 1988; Weiss et al., 1989a; Oikawa et al., 2005). A more recent study found that BMAA concentrations as low as 30 μM can cause selective death of motor neurons (Rao et al., 2006; Butt, 2011; Köles et al., 2011) and cholinergic neurons (Venance et al., 1997; Agulhon et al., 2008; Liu et al., 2010), and we found that BMAA concentrations as low as 10 μM can enhance neuronal death induced by amyloid-β or 1-methyl-4-phenylpyridinium ion (MPP+) (Seifert and Steinhäuser, 2001; Lobner et al., 2007).

Given the potential relevance of BMAA consumption to neurodegenerative diseases it is important to determine the mechanism of BMAA induced neuronal death. We have previously shown that BMAA induces neuronal death through three distinct mechanisms: activation of the NMDA and mGluR5 receptors, and induction of oxidative stress (Lobner et al., 2007; Benarroch, 2009). Through electrophysiological recording it has been shown that BMAA directly acts on NMDA receptors(Ross et al., 1987; Weiss and Choi, 1988; Brownson et al., 2002; Lobner et al., 2007; Poskanzer and Yuste, 2011). The current studies were designed to determine how BMAA activates mGluR5 receptors and induces oxidative stress. Evidence is presented that the cystine/glutamate antiporter (system x_C^-) plays an important role in these effects. System x_C^- involves the transport of cystine into the cell in exchange for glutamate being transported out of the cell. Given the functions of system x_C^- it seems likely that it plays an important role in neuronal survival and death. By releasing glutamate it can increase extracellular glutamate levels and potentially cause excitotoxicity. Through providing cystine uptake, it can regulate cellular glutathione levels and in this way determine whether oxidative stress induced neuronal death will occur. We find that the effects of BMAA on neuronal death involve actions on system x_C^- to both inhibit cystine uptake and increase glutamate release.

MATERIALS AND METHODS

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). ^{35}S-cystine was from Perkin Elmer Life and Analytical Sciences (Boston, MA). 5-(and -6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate

(carboxy-H2DCFDA) was from Molecular Probes (Eugene, OR).

Cortical cell cultures

Mixed cortical cell cultures containing neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Cornell-Bell et al., 1990; Lobner, 2000). Dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles's Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Cultures were maintained in humidified 5% CO_2 incubators at 37°C. Mice were handled in accordance with a protocol approved by our Institutional Animal Care Committee.

Induction of neuronal death

All experiments were performed on mixed cultures 13-15 days in vitro (DIV). Toxicity was induced by exposure to the toxic agents for 24 hours in media as described for plating except without serum. All exposure media contained 26 mM $NaHCO_3$, as it has been shown previously that HCO_3^- is required for expression of NMDA receptor mediated BMAA toxicity (Weiss and Choi, 1988; Fellin et al., 2006; Butt, 2011; Parpura et al., 2012).

Assay of neuronal death (LDH release)

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) release from damaged or destroyed cells, in the extracellular fluid

24 hours after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 μM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Bezzi et al., 1998; Lobner, 2000; Bezzi et al., 2004; Kreft et al., 2004) . Glial cell death (assessed by trypan blue staining) was not observed in any of the current studies. Therefore results are presented as percent neuronal death.

^{35}S-cystine uptake

Uptake of cystine was measured by exposure of cultures to ^{35}S-cystine (2 μCi/ml) for 20 min in the presence or absence of 3 mM BMAA and/or 1 mM S-4-carboxyphenyl glycine (CPG). Following the exposure to ^{35}S-cystine, the cultures were washed three times and dissolved in 1% SDS (250 μl). An aliquot (200 μl) was removed and added to scintillation fluid for counting. Values were normalized to control ^{35}S-cystine uptake (20 min exposure to ^{35}S-cystine without BMAA or CPG).

Glutathione assay

Total glutathione was assayed using a modification of a previous method (Baker et al., 1990; Duan et al., 2003; Lobner et al., 2003). Briefly, following exposure to BMAA for three hours, cells were washed with a HEPES buffered saline solution, dissolved in 200 μl of 1% salicylic acid, and centrifuged. A 25 μl aliquot of the supernatant was combined with 150 μl of 0.1 M phosphate/5 mM EDTA buffer, 10 μl of 20 mM dithiobis-2-nitrobenzoic acid, 100 μl of 5 mM NADPH, and 0.2 U of glutathione

reductase. Total glutathione was determined by kinetic analysis of absorbance changes at 402 nm for 1.5 min, with concentrations determined by comparison to a standard curve.

Assay of intracellular oxidative stress

Oxidative stress was assayed by measuring dichlorofluorescein oxidation using a fluorescent plate reader following a modification of a previous method (Wang and Joseph, 1999; Bridges et al., 2004; Lobner et al., 2007). Cultures were exposed to 3 mM BMAA for 3 hours in the presence of 5- (and -6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DFDA) (10 μM). The carboxy-H_2DFDA is de-esterified within cells to form a free acid that can then be oxidized to the fluorescent 2'7'-dichlorofluorecein (DCF). After the exposure to carboxy-H_2DCFDA, cultures were washed three times with culture media lacking serum. Fluorescence was then measured using a Fluoroskan Ascent fluorescence plate reader (Thermo lab systems). The excitation filter was set at 485 nm and emission filter at 538 nm. Background fluorescence (no carboxy-H_2DCFDA added) was subtracted and the results normalized to control conditions (carboxy-H_2DCFDA added, but no BMAA exposure).

Analysis of glutamate release

Glutamate release was measured following exposure to 3 mM BMAA or cystine for 1 hour. Experiments were performed in the presence of 10 μM MK-801 to block potential injury induced by glutamate release. Samples of the bathing media from the cell cultures were assayed for glutamate by using phenylisothiocyanate (PITC) derivatization, HPLC (Agilent 1100) separation using a Hypersil-ODS reverse phase

column, and ultraviolet detection at a wavelength of 254 nm (Cohen et al., 1986; Lobner and Choi, 1996; Magistretti and Pellerin, 1996; Edvinsson and Krause, 2002). 200 μl of the bathing media was derivatized with 100 μl of PITC, methanol, triethylamine (2,7,4) and dried under vacuum. These samples were then reconstituted in solvent consisting of 0.14 M sodium acetate, 0.05% TEA, 6% acetonitrile and brought to pH 6.4 with glacial acetic acid. The above solvent was used as the mobile phase with the column being washed between each sample run in 60% acetonitrile, 40% water. Media glutamate concentrations were calculated by normalizing to glutamate standards. Glutamate measurements were found to be linear over the range 0.1-10 μM.

Statistical analysis

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

RESULTS

BMAA toxicity involves the NMDA receptor, mGluR5 receptor, and oxidative stress

As has been shown before we found that blockade of NMDA receptors with MK-801 provided significant protection against high concentration BMAA toxicity (Ross et al., 1987; Weiss et al., 1989a; Dringen et al., 1993). As we have shown previously (Magistretti and Pellerin, 1996; Lobner et al., 2007) that the mGluR5 antagonist 6-methyl-2-[phenylethynel]-pyridine (MPEP), and the free radical scavenger, trolox, were not protective by themselves, but provided additional protection against BMAA toxicity

beyond that provided by MK-801 (Fig. 2.1.). Furthermore, the combination of these agents provided the greatest protection, suggesting that they were acting through distinct mechanisms. The MPEP and trolox were not protective against BMAA toxicity with the MK-801 present likely because of the overwhelming toxic effects of BMAA on NMDA receptor in the absence of MK-801 (Lobner et al., 2007). Activation of AMPA/kainate receptors does not appear to be involved as we have shown previously that blocking AMPA/kainate receptors with CNQX is not protective even in the presence of MK-801 (Freeman and Crapo, 1982; Simonian and Coyle, 1996; Lobner et al., 2007). A high concentration of BMAA (3 mM) was used so that complete neuronal death was induced and the different mechanisms of toxicity could be studied.

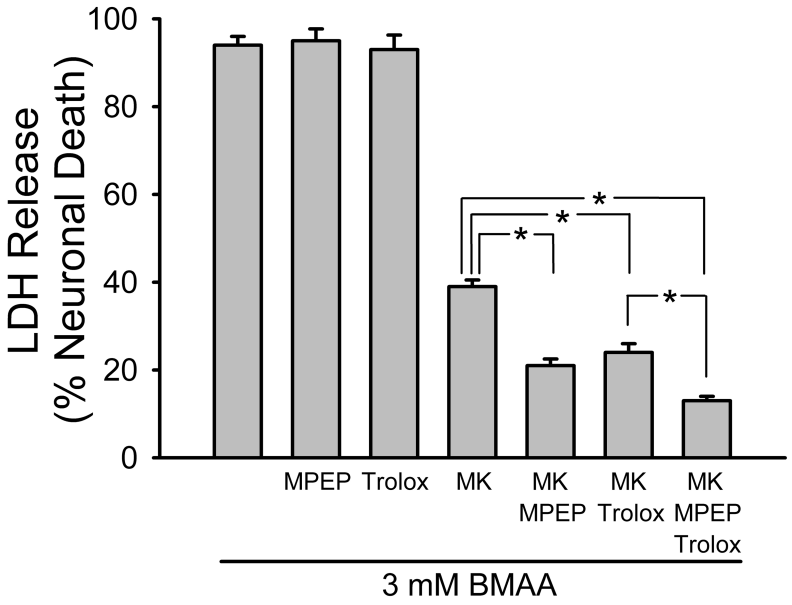

FIGURE 2.1. BMAA induced toxicity occurs through multiple mechanisms.

MK: 10 μM MK-801; MPEP: 50 μM 6-methyl-2-[phenylethynyl]-pyridine (MPEP); trolox: 100 μM trolox. Bars show % neuronal cell death (mean ± s.e.m., n = 16-20) quantified by measuring release of LDH, 24 hours after the beginning of the insult.
* indicates significant difference.

BMAA induces oxidative stress through inhibition of system x_C- mediated cystine uptake

One possibility for induction of oxidative stress by BMAA is that it inhibits cystine uptake. Cystine is the precursor for production of the endogenous free radical scavenger, glutathione. We found that BMAA did in fact greatly attenuate cystine uptake (Fig. 2.2.). Also, the cystine uptake was largely blocked by the cystine/glutamate antiporter (system x_C-) inhibitor (s)-4-carboxyphenylglycine (CPG), suggesting that themajority of the cystine uptake is system x_C- mediated. BMAA did not cause inhibition of uptake in the presence of CPG (Fig. 2.2.). Therefore, it is likely that BMAA attenuates the uptake of cystine by inhibition of system x_C-.

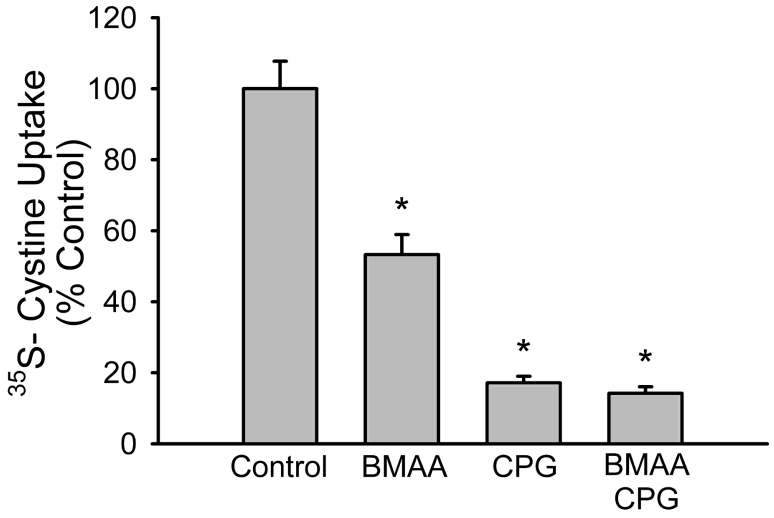

FIGURE 2.2. BMAA inhibits system x_C- mediated cystine uptake.
BMAA: 3 mM BMAA; CPG: 1 mM S-4-carboxyphenyl glycine (CPG). Bars show ^{35}S-cystine uptake during a 20 minute exposure presented as % control (mean ± s.e.m., n = 16). * indicates significant difference from control.

Since cystine uptake is rate-limiting step for glutathione synthesis, glutathione levels in response to BMAA treatment was measured. After a three hour treatment with BMAA, glutathione levels were decreased by about 50% (Fig. 2.3.). Three hour exposure was chosen because at this time point there was no significant neuronal death observed (less than 10% by the LDH release assay).

Since BMAA causes a decrease in the levels of the endogenous free radical scavenger glutathione, the total oxidative stress of the cells should be increased. We measured cellular oxidative stress with the fluorescent dye dichlorofluorescein (DCF). Treatment with BMAA for 3 hours caused a significant increase in oxidative stress, which was blocked by the free radical scavenger trolox (Fig. 2.4.).

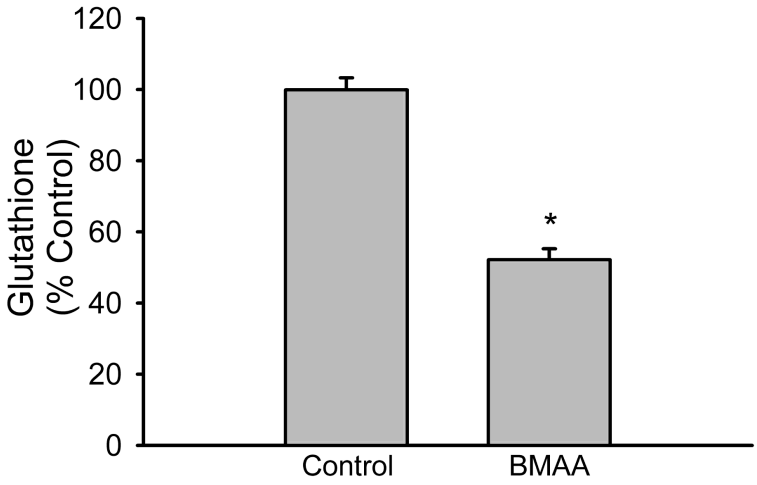

FIGURE 2.3. BMAA decreases cellular glutathione levels.

BMAA: 3 mM BMAA. Bars show glutathione levels following three hour BMAA exposure presented as % control (mean ± s.e.m, n = 16). * indicates significant difference from control.

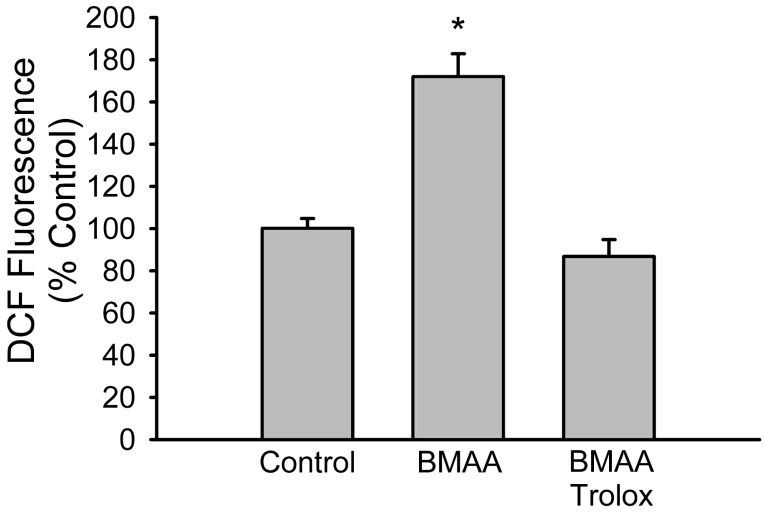

FIGURE 2.4. BMAA induces oxidative stress.

BMAA: 3 mM BMAA; trolox: 100 μM trolox. 5-(and -6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate (10 μM) was added to the cultures during a three hour exposure to BMAA. Bars show % control fluorescence (mean ± s.e.m., n = 12). * indicates significant difference from control.

The inhibition by BMAA of system x_C^- mediated cystine uptake suggests that BMAA competes with cystine; if like cystine, it is transported, the high levels of BMAA used in these studies should lead to increased glutamate release. We did in fact find that 3 mM BMAA increased extracellular glutamate. A similar increase was also caused by 3 mM cystine (Fig. 2.5.).

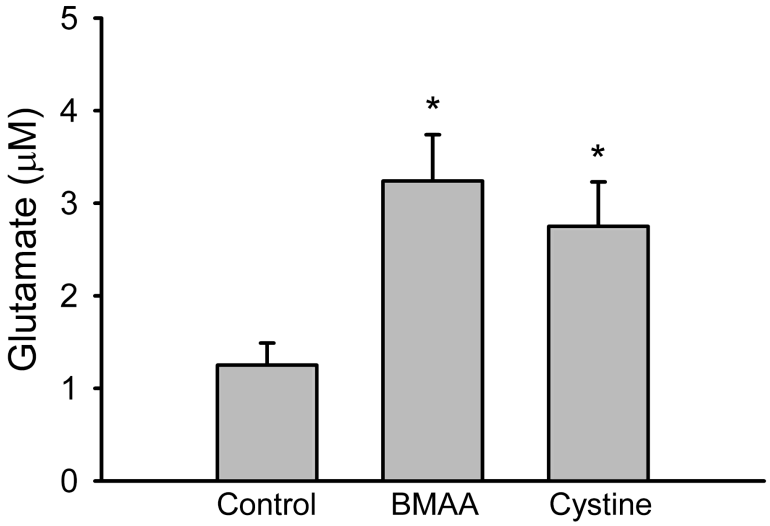

FIGURE 2.5. BMAA and cystine stimulate glutamate release.

BMAA: 3 mM BMAA; cystine: 3 mM cystine. Bars show extracellular glutamate levels following a 1 hour BMAA or cystine exposure (mean ± s.e.m., n = 8). * indicates significant difference from control.

Since BMAA appears to induce glutamate release via system x_C^-, it is possible that this glutamate is responsible for at least part of the glutamate receptor mediated neuronal death. Because BMAA is also a direct agonist for glutamate receptors (Lobner et al., 2007; Cucchiaroni et al., 2010), and 3 mM cystine caused a similar increase in extracellular glutamate compared with 3 mM BMAA, we used 3 mM cystine to study the effect of glutamate released by system x_C^-. We found that 3 mM cystine did induce neuronal death (Fig. 2.6.). However, the death was less than that caused by 3 mM BMAA, likely due to the fact that cystine is not a direct NMDA receptor agonist like BMAA. However, the cystine toxicity was partially blocked by either MPEP or MK-801. No protection was afforded by trolox, as would be expected since a high concentration of cystine should not induce oxidative stress.

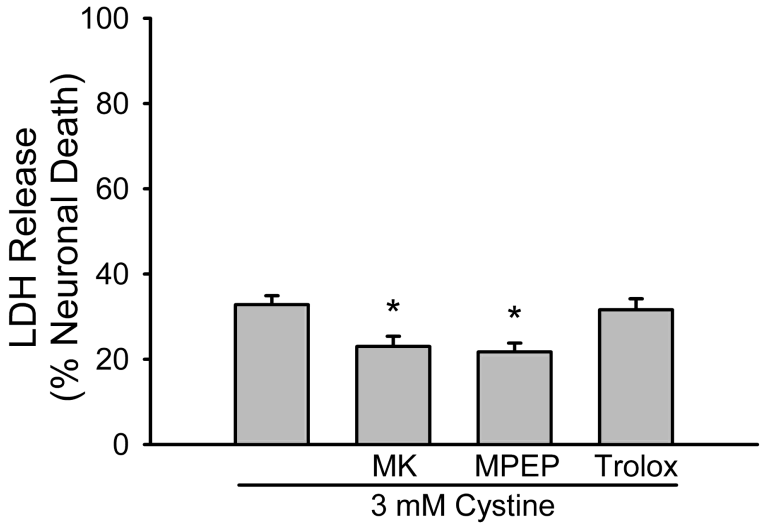

FIGURE 2.6. Cystine-induced neuronal death is attenuated by MK-801 and MPEP.
MK: 10 μM MK-801; MPEP: 50 μM MPEP; trolox: 100 μM trolox. Bars show % neuronal cell death (mean ± s.e.m, n = 24) quantified by measuring release of LDH, 24 hours after the beginning of the insult. * indicates significant difference from control (cystine alone).

DISCUSSION

We found that BMAA inhibits system x_C^- mediated cystine uptake leading to decreased cellular glutathione. The process of glutathione production in neurons involves a complex series of steps in which cystine is taken up primarily into astrocytes (Freeman and Crapo, 1982; Sagara et al., 1993). The glutathione produced by astrocytes is released and converted extracellularly into cysteine, which is taken up by neurons and used by them to produce glutathione (Fridovich, 1975; Wang and Cynader, 2000). Whether the action of BMAA to inhibit cystine uptake was primarily on neurons or astrocytes was not determined in this study. Therefore, it is not known if BMAA is initially acting to inhibit cystine uptake into neurons, or if it inhibits the uptake of cystine into astrocytes and prevents their release of glutathione and therefore restricts the supply of cysteine to neurons. Complicating this simple analysis of BMAA effects of glutathione levels is the

finding that blocking system xc- with CPG for as long as 24 hours does not cause a decrease in cellular glutathione levels (data not shown). The fact that BMAA does cause a decrease in cellular glutathione indicates that it has actions beyond its effect on system xc- mediated cystine uptake. Other known actions of BMAA that may play a role in its effect on glutathione levels include inhibition of the enzymes involved in glutathione synthesis (Esterhuizen-Londt et al., 2011), activation of NMDA and mGluR5 receptors (Lobner et al., 2007), and results presented in the current study indicating that it can cause glutamate release.

The data presented here strongly suggests that BMAA not only inhibits cystine uptake, but also drives system x_C- to release glutamate and that this glutamate induces neuronal death by acting on mGluR5 receptors and NMDA receptors. The evidence for this is that BMAA and cystine at the same concentration (3 mM) increase extracellular glutamate and they both induce NMDA receptor and mGluR5 mediated neuronal death. There is no evidence that either of these compounds directly activates mGluR5. Unfortunately, CPG could not be used to block the glutamate release because while it is a fairly poor substrate for system x_C- (Patel et al., 2004; Rush et al., 2012b), at the high concentrations needed to compete with the high concentrations of BMAA and cystine used in these studies it stimulated glutamate release by itself (data not shown). Why in our studies there was some protection against cystine induced toxicity by MK-801 is not clear. There is no published data indicating that cystine is a direct NMDA receptor agonist. However, we can not exclude the possibly that some of the cystine may be converted into cysteine, which is an NMDA receptor agonist (Pullan et al., 1987; Ballatori et al., 2009). Alternatively, the glutamate release stimulated by cystine via

system x_C^- may act to stimulate NMDA receptors. Furthermore, it has been shown activation of mGluR5 and NMDA receptor positively enhances each other's activity (Alagarsamy et al., 1999; Benquet et al., 2002; Chen et al., 2011), which could potentially add to the BMAA toxicity.

Both oxidative stress and excitotoxicity have been suggested to be regulated by system x_C^- in various cell types. It has been known for many years that inhibition of cystine uptake mediated by system x_C^- can induce neuronal death by decreasing glutathione levels (Murphy et al., 1989; Murphy and Baraban, 1990; Dringen et al., 2000) In contrast, astrocytes activated by IL-beta were shown to kill cortical neurons by release glutamate via system x_C^- (Bolaños et al., 1995; Rice and Russo-Menna, 1998; Fogal et al., 2007). Also, microglia can release glutamate via system x_C^- that can kill cerebellar granule cells (Deneke and Fanburg, 1989; Piani and Fontana, 1994), oligodendrocytes (Rahman et al., 2005; Domercq et al., 2007), and enhance amyloid-β induced neuronal death in cortical cultures (Beutler, 1989; Deneke and Fanburg, 1989; Qin et al., 2006a). Finally, glutamate release from kidney dendritic cells via system x_C^- can inhibit T cell activation through actions on mGluR5 receptors (McBean, 2002; Pacheco et al., 2006). In the current study we find evidence that oxidative stress induced by the inhibition of cystine uptake and excitotoxicity induced by system x_C^- mediated glutamate release both play a role in BMAA induced neuronal death.

Interestingly, another amino acid implicated in neurological diseases, β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), also shares some properties with BMAA: it directly activates glutamatergic receptors and is transported by system x_C^- (Kranich et al., 1996; Chase et al., 2007). It is likely that ODAP acts similar to BMAA and causes

cytotoxicity through at least some of the same mechanisms.

The finding that BMAA not only inhibited cystine uptake by system x_C^-, but also stimulated glutamate release, suggests that it was transported by system x_C^-. This transport provides a mechanism by which BMAA can accumulate in cells. There is evidence that BMAA may be incorporated into proteins (Murch et al., 2004b; Lobner, 2009) and could therefore potentially play a role in the protein misfolding found in neurodegenerative diseases (McBean, 2002; Uversky, 2008). Whether BMAA can be incorporated into GSH is unknown. If BMAA does become incorporated into GSH this would provide another potential mechanism for its ability to cause depletion of GSH and oxidative stress.

The importance of BMAA acting on system x_C^- to induce oxidative stress and excitotoxicity at the same time is that this may be partially account for the different types of neurological diseases that have been associated with BMAA consumption. It is possible that the different actions of BMAA, in association with varying underlining conditions, may lead to the multiple disorders. Most neurodegenerative diseases appear to involve NMDA receptor mediated excitotoxicity (Arrick et al., 1985; Lipton and Chen, 2004) and oxidative stress (Dringen et al., 1999; Cui et al., 2004), and mGluR5 receptor activation has been implicated in Parkinson's disease as well (Zerangue and Kavanaugh, 1996b; Chen and Swanson, 2003; Marino et al., 2003; Aoyama et al., 2006). All of these mechanisms of neuronal injury may be enhanced by BMAA. The finding that BMAA causes a depletion of cellular glutathione is of particular importance. There is evidence that glutathione depletion plays a role in Alzheimer's disease, Parkinson's disease, and ALS (Bannai and Kitamura, 1980; Bains and Shaw, 1997; Liu et al., 2004; Zeevalk et al.,

2008). This type of low level, long-term, depletion of glutathione that may be caused by the inhibition of cystine uptake could cause the long-term oxidative stress that may underlie aspects of neurodegenerative diseases.

From these results and previous studies, it is obvious that system x_C^- plays an important role in neuronal fate. We next want to examine if system x_C^- can be regulated by neurotrophic factors, which are naturally occurring molecules, which also regulate neuronal fate in multiple ways. Neurotrophic factors have wide ranging effects in the central nervous system, including altering excitotoxicity (Koh et al., 1995; Sato et al., 1999), oxidative stress (Zhang et al., 1993).

CHAPTER III

FUNCTIONAL UPREGULATION OF SYSTEM XC- BY FIBROBLAST GROWTH FACTOR-2

ABSTRACT

The cystine/glutamate antiporter (system x_C^-) is a sodium-independent amino acid transport system. Disruption of this system may lead to multiple effects in the central nervous system including decreased cellular glutathione. Since multiple neurological diseases involve glutathione depletion, and disruption of growth factor signaling has also been implicated in these diseases, it is possible that some growth factors effects are mediated by regulation of system x_C^-. We tested the growth factors fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), neuregulin-1 (NRG), neurotrophin-4 (NT-4), and brain derived neurotrophic factor (BDNF) on system x_C^- mediated ^{14}C-cystine uptake in mixed neuronal and glial cortical cultures. Only FGF-2 significantly increased cystine uptake. The effect was observed in astrocyte-enriched cultures, but not in cultures of neurons or microglia. The increase was blocked by the system x_C^- inhibitor (s)-4-carboxyphenylglycine (CPG), required at least 12 hour FGF-2 treatment, and was prevented by the protein synthesis inhibitor cycloheximide. Kinetic analysis indicated FGF-2 treatment increased the V_{max} for cystine uptake while the K_m remained the same. Quantitative PCR showed an increase in mRNA for xCT, the functional subunit of system x_C^-, beginning at 3 hours of FGF-2 treatment, with a dramatic increase after 12 hours. Blocking FGFR1 with PD 166866 blocked the FGF-2 effect. Treatment with both a PI3-kinase inhibitor (LY-294002) or a MEK/ERK inhibitor (U0126) for 1 hour prior to and during the FGF-2 treatment blocked the increased cystine uptake. The upregulation of system x_C^- by FGF-2 may be responsible for some of the known physiological actions of FGF-2.

INTRODUCTION

The function of system x_C^- makes it likely to play an important role in regulating many processes in the central nervous system. By releasing glutamate, it can increase extracellular glutamate levels, which may alter neuronal plasticity and potentially cause excitotoxicity; Through providing cystine uptake, it supplies the substrate for cellular glutathione synthesis, and in this way may determine whether oxidative stress induced neuronal death will occur.

There is considerable evidence that glutamate released from system x_C^- is involved in both physiological and pathological processes. Physiologically glutamate release from system x_C^- plays a role at the photoreceptor synapse (Hu et al., 2008) and in regulating immune cell function (Xue and Field, 2011). It is known that glutamate release from system x_C^- can cause toxicity, and this is observed in many pathological processes. Activated astrocytes release glutamate via system x_C^- that can kill cortical neurons (Fogal et al., 2007). Also activated microglia release glutamate via system x_C^- that can kill cerebellar granule cells (Piani and Fontana, 1994) and enhance amyloid-beta induced neuronal death in cortical cultures (Qin et al., 2006b).

Glutathione is known to detoxify superoxide in a reaction catalyzed by glutathione peroxidase forming disulfide/oxidized glutathione. This reaction is accomplished by redox changes at the sulfhydryl group of the cysteine residue of glutathione (Dringen and Hirrlinger, 2003). The cystine uptake mediated by system x_C^- appears to play a critical role in regulating cellular glutathione levels, because de novo synthesis of glutathione in the brain is rate-limited by the uptake of cystine (Sagara et al., 1993). Specifically, astrocytes express high levels of system x_C^- and have high levels of

cystine uptake (Dringen et al., 2000; Lewerenz et al., 2006), while neurons of the central nervous system have limited capacity for cystine uptake, and are reliant primarily on astrocytes for the provision of cysteine and cysteinylglycine for neuronal glutathione production (Dringen et al., 1999). The first evidence that system x_C^- could play a role in neuronal death was provided when it was shown that high concentrations of glutamate could competitively inhibit cystine uptake leading to an oxidative stress mediated neuronal death (Murphy et al., 1990). This type of toxicity was shown to occur before glutamate receptors developed (Murphy and Baraban, 1990) and to involve depletion of glutathione (Ratan et al., 1994). In contrast to the enhancement of neurotoxicity caused by releasing glutamate, system x_C^- activity on non-neuronal cells can be protective to neurons by generating and releasing glutathione. Overexpression of xCT, the functional subunit of system x_C^-, in astrocytes has been shown to enhance glutathione release and protect neurons from oxidative stress (Shih et al., 2006). Since neurological diseases including schizophrenia, depression, Alzheimer's disease, and Parkinson's disease have all been associated with depletion of cellular glutathione (Ballatori et al., 2009; Dean et al., 2009; Shibasaki et al., 2009; Maes et al., 2011), it is possible that altered system x_C^- function plays a role in these diseases.

Neurotrophic factors have wide ranging effects in the central nervous system including altering excitotoxicity (Koh et al., 1995; Chintala et al., 2005), oxidative stress (Zhang et al., 1993; Shih et al., 2006), and cellular glutathione levels (Pan and Perez-Polo, 1993; Sato et al., 1999). The overlapping actions of growth factors and system x_C^- suggest the possibility that some neurotrophic factor effects may be mediated by

regulation of system x_C^-. Therefore we tested the effects of a number of well-established neurotrophic factors on system x_C^- function.

MATERIALS AND METHODS

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). Serum was from Atlanta Biologicals (Lawrenceville, GA). Growth factors were from Alomone Labs (Jerusalem, Israel). ^{14}C-cystine was from Perkin Elmer (Waltham, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000; Sasaki et al., 2002). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Neuron-enriched cultures were prepared exactly as above with the addition of 10 μM cytosine arabinoside 48 hours after plating to inhibit glial replication. In these cultures <1% of cells are astrocytes (Dugan et al., 1995; Sato et al., 2000; Sasaki et al., 2002; Rush et al., 2010). Astrocyte-enriched cultures were prepared as described for mixed cultures except they are from cortical tissue taken from post-natal day 1-3 mice (Choi et al., 1987; Schwartz and Wilson, 1992;

Venugopal and Jaiswal, 1996; Rush et al., 2010). Microglia cultures were prepared by first growing astrocyte-enriched cultures, then shaking the microglia free and plating them in media containing 10 ng/ml colony stimulating factor (CSF) (Itoh et al., 1999; Barger and Basile, 2001). Cultures were maintained in humidified 5% CO_2 incubators at 37°C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Heath Service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

^{14}C-cystine uptake

System x_C^- mediated uptake of radiolabeled cystine was assayed as previously described with modifications (Itoh et al., 1999; Liu et al., 2009). Growth factors were added to the serum containing media for the indicated durations. Cultures were then washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025μCi/ml) for 20 min. Following ^{14}C-cystine exposure, cultures were washed with ice cold HEPES buffered saline solution and dissolved in 250 μl sodium dodecyl sulfate (0.1%). An aliquot (200 μl) was removed and added to scintillation fluid for counting. In some experiments an aliquot was also used to measure cellular protein levels by the BCA assay. Values were normalized to ^{14}C-cystine uptake in untreated control on the same experimental plate. Kinetic analysis of cystine uptake was performed as previously described (Lee and Johnson, 2004; Fogal et al., 2007). Uptake of ^{14}C-cystine was measured over a range of cystine concentrations: 0.1 μM, 0.3 μM, 1μM, 3 μM, 10 μM, and 30 μM. Kinetic analysis was performed using the Hanes-Woolf plot where

[substrate]/velocity is plotted against [substrate]. The slope corresponds to $1/V_{max}$, while the X-intercept corresponds to the K_m.

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

Transcriptional expression of xCT in astrocyte-enriched cultures after stimulation with FGF-2 was examined using RT-qPCR. Briefly, after various durations of treatment with FGF-2 cells were washed 3 times with cold PBS. Total RNA was isolated with TRIzol extraction (Invitrogen; Carlsbad, CA) according to manufacturer's protocol, cDNA was synthesized using the Reverse Transcription System (Promega; Madison, WI) from 1 μg of total RNA following the manufacturer's protocol. All products were amplified on a StepOne real-time PCR system (Applied Biosystems; Carlsbad, CA) using 100 ng of cDNA, PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersberg, MD), and the following primers: gapdh mouse forward-AAG GGC TCA TGA CCA CAG TC; gapdh mouse reverse-GGA TAC AGG GAT GAT GTT CT; xCT mouse forward-AGG GCA TAC TCC AGA ACA CG; xCT mouse reverse-GAC AGG GCT CCA AAA AGT GA. xCT expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (gapdh). Quantitative PCR was used to determine fold increase of xCT mRNA using the $2^{-\Delta\Delta C}{}_T$ method.

Statistical analysis

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

RESULTS

The effects of FGF-2, IGF-1, NRG, NT-4, and BDNF, each of which have been shown to have actions on cortical cells (Bannai, 1984; Koh et al., 1995; Anton et al., 1997; Erickson et al., 2002; Sasaki et al., 2002; Lobner et al., 2003), were tested on ^{14}C-cystine uptake in mixed neuronal and glial cortical cultures. In these cultures approximately 90% of cystine uptake is mediated by system x_C^- (Lee and Johnson, 2004; Liu et al., 2009; Lobner, 2009). Following 24 hour growth factor treatment, only FGF-2 caused a significant increase in cystine uptake (Fig. 3.1.). Importantly, the growth factors were added to the cultures on DIV 13-14, a time point at which there was already a confluent layer of astrocytes. None of the growth factors had a significant effect on total cellular protein levels (data not shown).

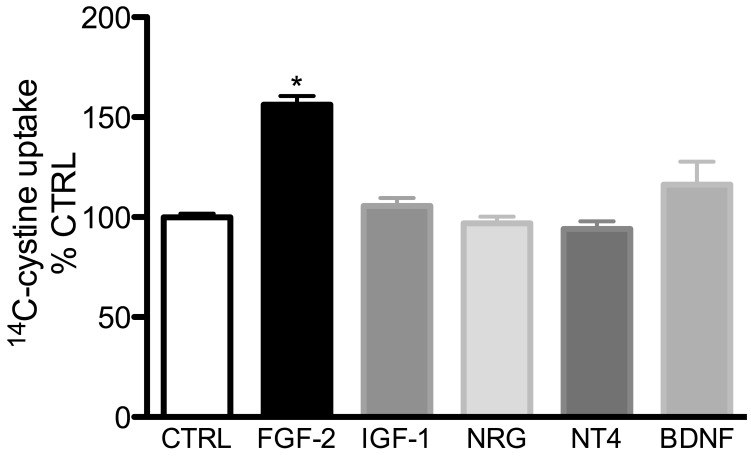

FIGURE 3.1. Growth factor effects on cystine uptake in cortical cultures.

Treatment for 24 hours with 100 ng/ml of the growth factors on mixed neuronal and glial cultures. FGF-2: fibroblast growth factor-2; IGF-1: insulin-like growth factor-1; NRG: neuregulin; NT-4: neurotrophin-4; BDNF: brain derived neurotrophic factor. Cultures were exposed to growth factors for 24 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean ± s.e.m., n = 12-16). * indicates significantly different from control uptake.

This effect of FGF-2 on cystine uptake does not appear to be limited to cortical astrocytes. We also examined some growth factors that are known to have effects on spinal cord, and found a similar regulatory effect of FGF-2 on cystine uptake in spinal cord glial cultures (Fig. 3.2.).

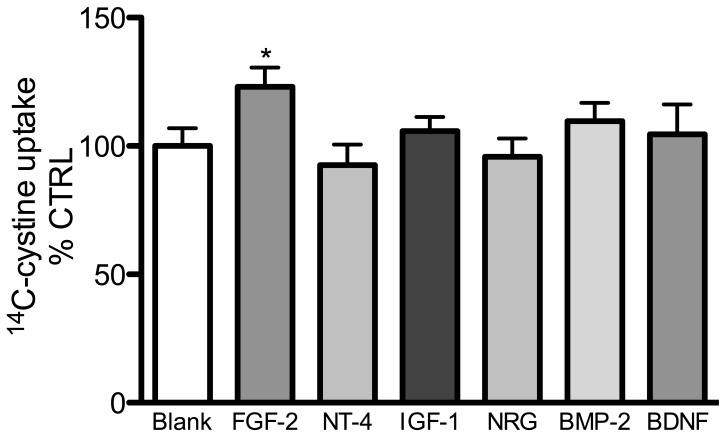

FIGURE 3.2. Growth factor effects on cystine uptake spinal cord glial cultures. 24 hour treatment with growth factors (100 ng/ml) on primary mouse spinal cord glial cultures. FGF-2: fibroblast growth factor-2; NT-4: neurotrophin-4; IGF-1: insulin-like growth factor-1; NRG: neuregulin; BMP-2: Bone morphogenetic protein 2; BDNF: brain derived neurotrophic factor. Cultures were exposed to growth factors for 24 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min. Spinal cord glia cultures were prepared as cortical glial cultures except they were prepared from whole spinal cords from postnatal day one mice. Bars show % control (mean±s.e.m., n=12-16). * indicates significantly different from control uptake.

We also examined FGF-2 actions on neuronal-enriched, astrocyte-enriched, and microglial cultures to see which cell type mediates the FGF-2 effect. We found that the FGF-2 effect only occurred in astrocyte-enriched cultures (Fig. 3.3.). We have shown previously that the majority of cystine uptake in cortical cultures is into the glial cells (Lobner, 2009). Astrocyte-enriched cultures prepared by the current method are known to also contain microglial cells (Shih et al., 2003; Hamby et al., 2006; Wang et al., 2009).

However, since there was no effect of FGF-2 in the microglial cultures, we believe that the effect occurs on astrocytes. Therefore, all of the following experiments were performed using the astrocyte-enriched cultures. We next tested whether the effect of FGF-2 was on system x_C^- mediated cystine uptake. More than 90% of cystine uptake was blocked by the system x_C^- antagonist (s)-4-carboxyphenylglycine (CPG), and the increase caused by 24 hour treatment with FGF-2 was completely blocked by CPG (Fig. 3.4.).

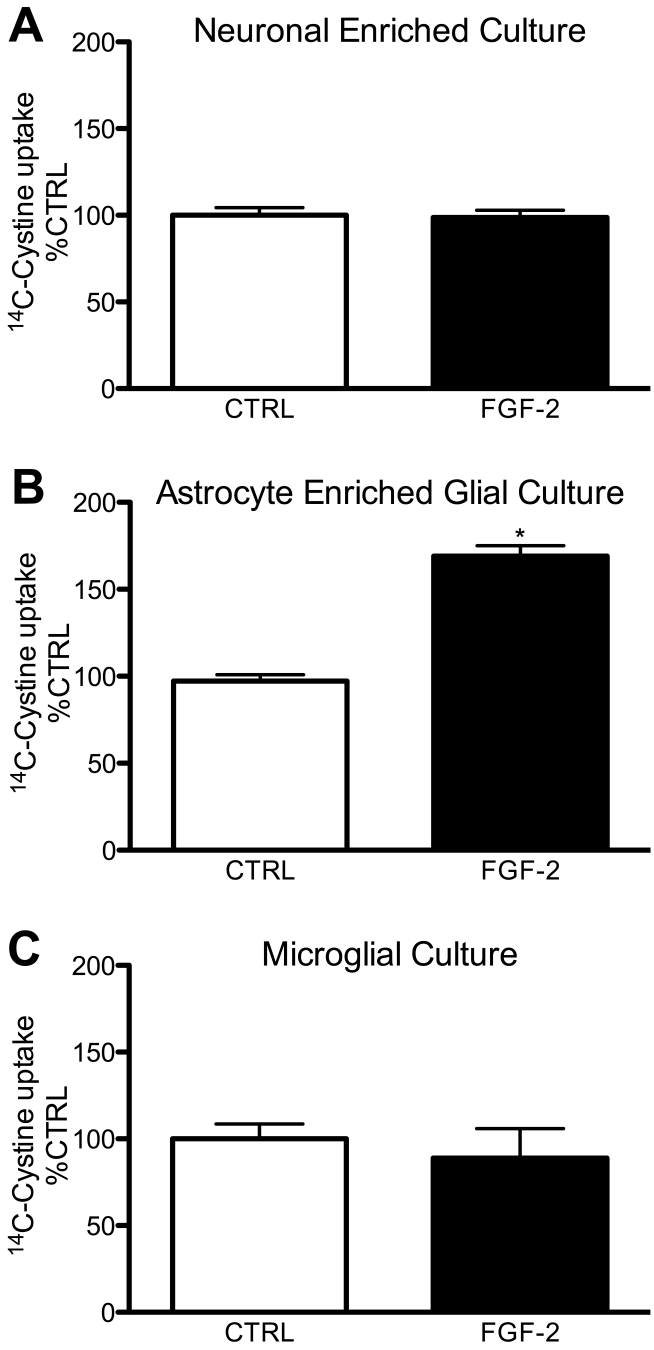

FIGURE 3.3. Effects of FGF-2 on cystine uptake in neuronal-enriched cultures (A), astrocyte-enriched cultures (B), and microglial cultures (C).

Cultures were exposed to FGF-2 (100 ng/ml) for 24 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min. Bar show % control (mean±s.e.m., n=8). * indicates significantly different from control uptake.

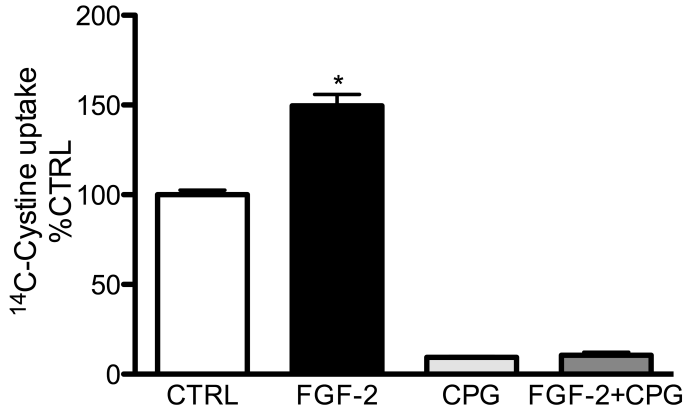

FIGURE 3.4. FGF-2 induced increase in cystine uptake is mediated by system x_C^-.

Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 24 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min with or without the system x_C^- antagonist (s)-4-carboxyphenylglycine (200 μM CPG). Bars show % control (mean±s.e.m., n=8). * indicates significantly different from control uptake.

The previous studies were all performed using a 24 hour growth factor treatment. We next performed a time course of FGF-2 treatment. A significant effect of FGF-2 treatment was first observed after a 12 hour exposure (Fig. 3.5A.). None of the growth factors tested in Fig. 3.1., including FGF-2, had an effect with acute treatment (20 min prior to and during the cystine uptake-data not shown). Treatment with the protein synthesis inhibitor cycloheximide during the 24 hour FGF-2 treatment blocked the FGF-2 induced increase in cystine uptake (Fig. 3.5B.). These results suggest an upregulation of the number of transporters.

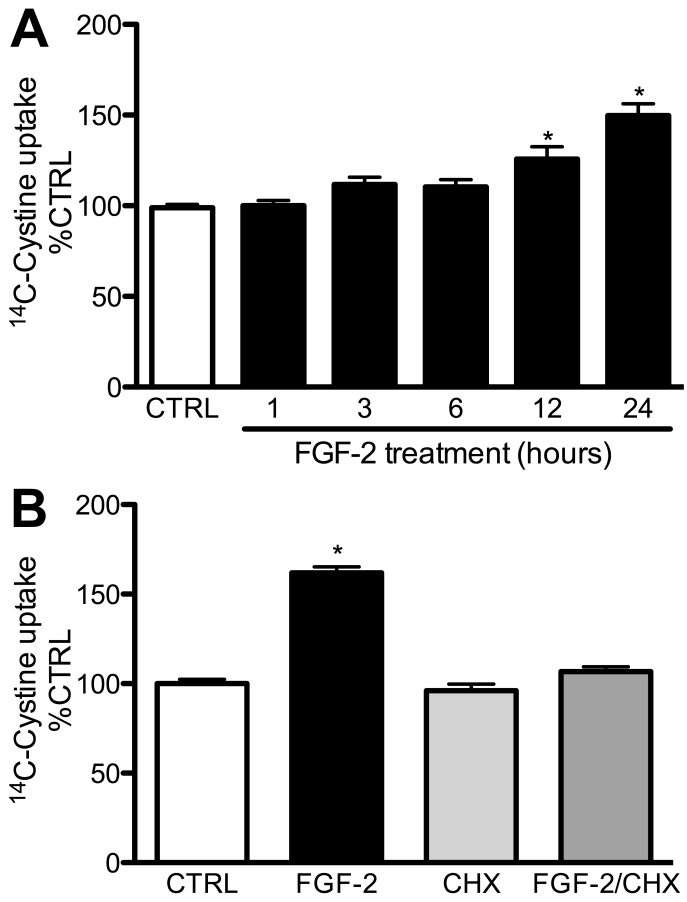

FIGURE 3.4. Time and protein synthesis dependent changes in cystine uptake induced by FGF-2 treatment on astrocyte-enriched cultures.

(A) Cultures were exposed to FGF-2 (100 ng/ml) for the indicated period of time, washed into a growth factor free media and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean±s.e.m., n=32). (B) Cultures were exposed to FGF-2 (100 ng/ml) for 24 hours with or without the protein synthesis inhibitor cycloheximide (CHX, 200 ng/ml), washed into a growth factor and inhibitor free media, and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean±s.e.m., n=8). * indicates significantly different from control uptake.

We also assessed the kinetic changes of the transporter. Treatment with FGF-2 for 24 hours caused a significant increase in the V_{max} of the transporter activity, but no change in the K_m (Fig. 3.6.).

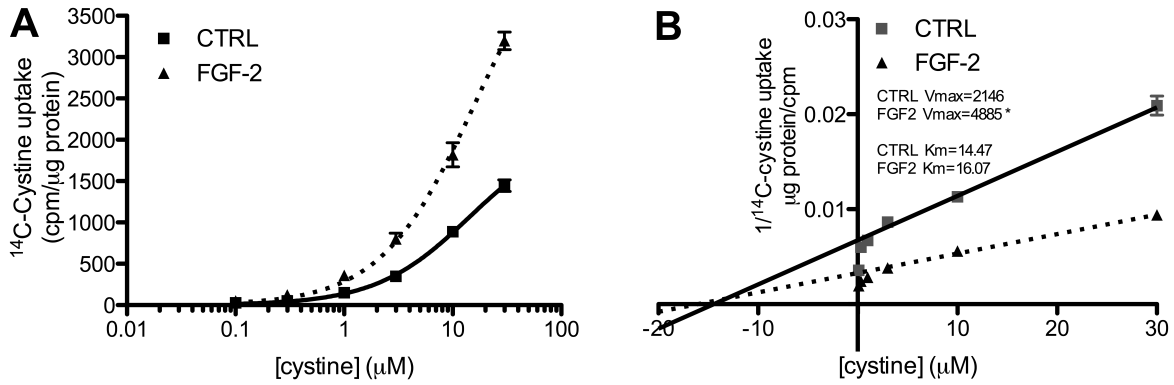

FIGURE 3.6. Kinetic analysis of FGF-2 effects on cystine uptake.

Following 24 hour exposure to FGF-2 (100 ng/ml), ^{14}C-cystine uptake was measured for 20 min in astrocyte-enriched cultures in the presence of varying concentrations of cystine. Data are expressed as ^{14}C-cystine uptake in CPM per microgram protein (mean±s.e.m., n=4) (A). V_{max} (1/slope) and K_m (-X-intercept) were determined using the Hanes-Woolf plot (B). * indicates significantly different from control uptake.

Finally, a time course of mRNA levels of xCT, the functional subunit of system x_C-, measured with RT-qPCR, showed an increase starting after 3 hours of FGF-2 treatment, with a large increase after 12 hours (Fig. 3.7.).

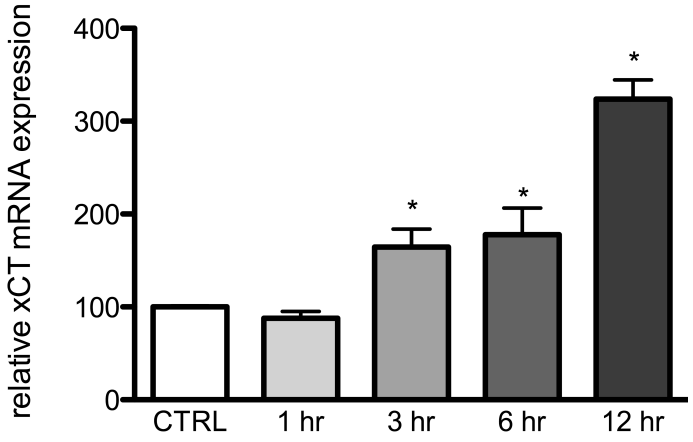

FIGURE 3.7. Time course of changes in xCT mRNA in astrocyte-enriched cultures assessed by RT-qPCR following different durations of FGF-2 (100 ng/ml) treatment.

Bars show % control (mean ± s.e.m.), data is from 4 independent experiments run in duplicate for n=8. * indicates significantly different from control.

FGF-2 can act on a number of FGF receptors. FGFR1 is highly expressed in the brain (Gonzalez et al., 1995; Wang et al., 2009) and it is found at high levels in astrocytes in the current culture system (Lobner and Ali, 2002; Sato et al., 2004; Lewerenz and Maher, 2009). Therefore we tested the effects of an antagonist of this receptor on the FGF-2 treatment induced increase in system x_C^- function. The FGFR1 antagonist PD 166866 caused a concentration dependent inhibition of FGF-2 stimulated cystine uptake (Fig. 3.8A.). We next attempted to determine the signaling pathway of the FGF-2 effect. We tested the effects of selective inhibitors of the MEK/ERK (U0126) (Favata et al., 1998; Kilberg et al., 2005) and PI3-kinase (Vlahos et al., 1994) pathways on the FGF-2 stimulated cystine uptake. Inhibition of either the MEK/ERK or PI3-kinase pathway partially blocked the FGF-2 stimulated cystine uptake, while the combination of the inhibitors completely blocked the increase (Fig. 3.8B.).

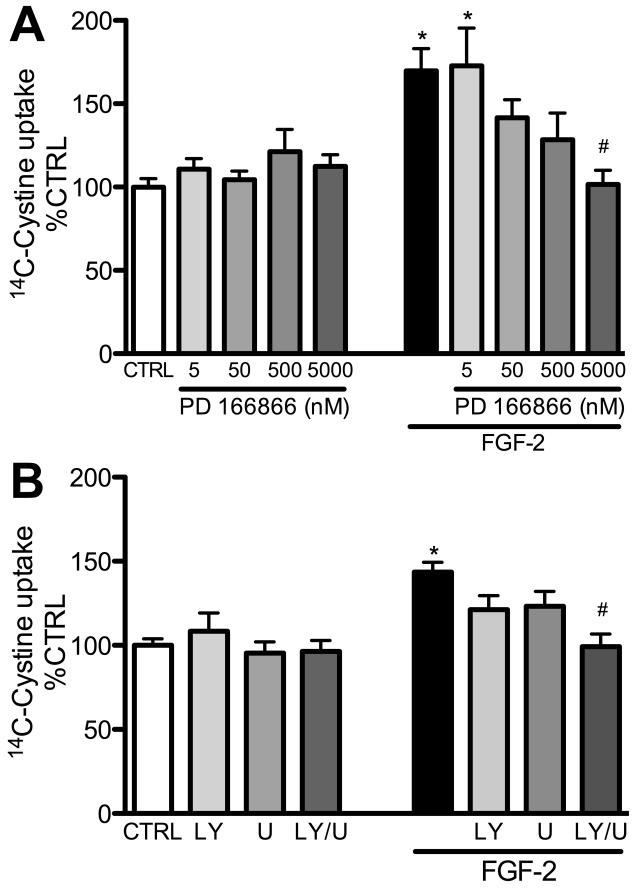

FIGURE 3.8. FGF-2 stimulation of cystine uptake is mediated by activation of FGFR1 and both the MEK/ERK and PI3-kinase pathways.

(A) Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 24 hours with or without the FGFR1 inhibitor (PD 166866), washed into a growth factor and inhibitor free media, and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean±s.e.m., n=12). (B) Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 24 hours with or without the MEK/ERK inhibitor (10 μM U0126) or the PI3-kinase inhibitor (45 μM LY-294002) added 1 hour prior to and during the FGF-2 exposure, washed into a growth factor and inhibitor free media, and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean ± s.e.m., n = 18). * indicates significantly different from control uptake. # indicates significantly different from FGF-2 stimulated uptake.

DISCUSSION

The upregulation of system x_C^- induced by the FGF-2 treatment has multiple implications for understanding the biological effects of FGF-2 and its potential use as a therapeutic agent. Both dysregulation of FGF-2 signaling and system x_C^- function have

been implicated in psychiatric disorders such as schizophrenia, depression, and addiction. As FGF-2 and regulators for system x_C^- have been suggested for therapeutic use, it is important to understand the mechanism of regulation of system x_C^-. Also, upregulation of system x_C^- must be taken into account when considering the use of FGF-2 for the treatment of neurological conditions, particularly those that involve excitotoxicity.

The evidence suggesting the involvement of dysregulation of the FGF-2 system in schizophrenia is substantial (Terwisscha van Scheltinga et al., 2010). FGF-2 is involved in neurogenesis and normal brain development (Zechel et al., 2010). FGFR1 is widely expressed in the brain (Gonzalez et al., 1995) and is required for proliferation of hippocampal stem cells (Ohkubo et al., 2004). Therefore FGF-2 system signaling dysregulation may be involved in altered development in schizophrenia. Supporting this idea, in post-mortem brains from schizophrenia patients there was found to be increased FGFR1 expression in the CA4 region of the hippocampus (Gaughran et al., 2006) and there is increased expression of FGF-2 in the serum of schizophrenia patients (Hashimoto et al., 2003). It is interesting to note that disruption of MEK/ERK and PI3-kinase signaling have also been implicated in developmental diseases, such as schizophrenia (Waite and Eickholt, 2010; Yuan et al., 2010). We found that the action of FGF-2 to upregulate system x_C^- function was partially dependent on both MEK/ERK and PI3-kinase signaling. The results presented in the current study of FGF-2 effects on system x_C^- that are mediated by FGFR1 provide a potential mechanism by which defects in FGF-2 signaling leads to developmental disorders.

There are decreased levels of glutathione in schizophrenics (Raffa et al., 2011) and an animal model of schizophrenia involves the post-natal treatment with buthionine

sulfoximine, an inhibitor of a glutathione synthesis (Cabungcal et al., 2007). In one study it was found that in an animal model of schizophrenia there was decreased system x_C- activity, although in the brain of human schizophrenics there was a slight increase in xCT expression (Baker et al., 2008). Therefore, it is possible that increased FGF-2 signaling is a compensatory mechanism in schizophrenia to increase xCT expression to increase system x_C- activity. However, a great deal more work determining the time course of changes in FGF-2 system signaling, system x_C- function, glutathione levels, development changes, and development of the disease is necessary.

Disruption of FGF-2 signaling may also be involved in depression. In the post-mortem brains of depressed patients there is reduced mRNA for FGF-2, but increased mRNA for FGFR1 (Gaughran et al., 2006). Treatment with FGF-2 has antidepressant effects in mouse models of depression and reversed the decrease in neurogenesis in the hippocampus of these mice (Zechel et al., 2010).

Alterations in both system x_C- and FGF-2 have been implicated in cocaine addiction. In adult rats, following withdrawal from repeated cocaine, there is decreased function of system x_C- in the nucleus accumbens (Baker et al., 2003). Repeated cocaine administration increases FGF-2 mRNA in the striatum and prefrontal cortex (Fumagalli et al., 2006), while it decreases FGFR1 mRNA in the hippocampus but increases it in the prefrontal cortex (Turner et al., 2008b). Due to differences in the areas of the brain assessed, it is at this time not possible to draw conclusions regarding the interaction between system x_C- and FGF-2 during addiction. However, it is clear that both are changed.

We showed that 24 hour treatment with FGF-2 increases cystine uptake through system x_C^- selectively on primary cortical astrocytes, but not neurons or microglia. This effect does not appear to be limited to cortical astrocytes. We found a similar regulatory effect of FGF-2 on cystine uptake through system x_C^- in spinal cord glial cultures. The result that FGF-2 also increases cystine uptake in spinal cord gial cultures is especially interesting because of a recent study showing that knocking out FGF-2 can delay disease progression in the SOD1 ALS mouse model (Thau et al., 2012). One possible explanation for this result is that the lack of FGF-2 signaling leads to decreased system x_C^- activity in the ALS mice, and the protection would then be due to decreased glutamate release from system x_C^- and decreased excitotoxicity. However, what role FGF-2 plays in ALS is unclear. Post mortem studies revealed that there was no change in FGF-2 or FGFR1 mRNA in ALS patients compared to control (Petri et al., 2009). Preliminary data from our laboratory on mutant SOD1 mice showed a significant increase in system x_C^- function in spinal cord slices even before the animals showed any symptoms (unpublished data). Future studies on FGF-2 signaling and regulation of system x_C^- in the SOD1 model can potentially help clarify the involvement of FGF-2 and system x_C^- in ALS.

The function of system x_C^- makes it likely to play an important role in regulating neuronal survival and death; by releasing glutamate it can increase extracellular glutamate levels and potentially cause excitotoxicity, through providing cystine to the cell it regulates cellular glutathione levels and in this way may protect cells against oxidative stress. Both excitotoxicity and oxidative stress play a role in neurodegenerative diseases (ALS) (Bains and Shaw, 1997; Lau and Tymianski, 2010). The dual nature of system x_C^-

means that it may either protect against or enhance neuronal death depending on the relative importance of excitotoxicity and oxidative stress in each disease.

FGF-2 was tested in clinical trials for the treatment of stroke. It was found that FGF-2 treatment caused roughly a doubling of the mortality rate (Clark et al., 2000; Bogousslavsky et al., 2002). While there are many possible reasons for a lack of protective effects of FGF-2, the increase in mortality indicates that, in this situation, it was having injury potentiating effects. Could an increase in system x_C^- be responsible for the increased mortality following FGF-2 treatment? Certainly excitotoxicity plays a role in cerebral ischemia (Choi, 1998) and system x_C^- activity can enhance excitotoxicity (Fogal et al., 2007). However, FGF-2 has multiple effects on systems that can alter neuronal death. For example, in the central nervous system, it increases AMPA receptor expression (Cheng et al., 1995), and decreases NMDA receptor expression (Mattson et al., 1993). Therefore, the net effect of FGF-2 on neuronal death in various conditions will depend on many factors. We began to explore the net effect of FGF-2 by testing whether long-term upregulation of system x_C^- by FGF-2 directly induces neuronal death.

CHAPTER IV

FGF-2 INDUCES NEURONAL DEATH

ABSTRACT

FGF-2 upregulates system x_C^- selectively on astrocytes, which will lead to both increased cystine uptake, with increased glutathione production, and increased extracellular glutamate. While the increased intracellular glutathione can contribute to decreased oxidative stress, the increased glutamate release can potentially lead to excitotoxicity to neurons. To test this hypothesis, we performed a long-term treatment (48 hours) of FGF-2 in mature mixed neuronal and glial cultures. Treatment with FGF-2 for 48 hours caused a significant neuronal death in these cultures. This cell death was not observed in neuronal-enriched cultures, or astrocyte-enriched cultures, suggesting the toxicity is a result of neuron-glia interaction. Blocking system x_C^- eliminated the neuronal death. Since increasing cystine uptake is unlikely to induce toxicity, we examined the involvement of glutamate release. The general AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) abolished the neuronal toxicity induced by FGF-2 treatment, and surprisingly, the NMDA receptor antagonist memantine did not. When cultures were exposed directly to glutamate, either NBQX or memantine blocked the neuronal toxicity. The mechanism of this altered profile of glutamate receptor mediated toxicity by FGF-2 is unclear. The selective calcium permeable AMPA receptor antagonist 1-naphthyl acetyl spermine (NASPM) failed to offer protection. FGF-2 has been suggested to be able to decrease NMDA receptor mediated toxicity by both reducing NMDA receptor expression and causing a faster inactivation. FGF-2 has also been shown to increase GluR1 expression, as well as increased calcium influx. Therefore, the most logical explanation for the results is that 48 hour FGF-2 treatment induces AMPA receptor toxicity through

increased system x_C^- function resulting in increased release of glutamate. At the same time, FGF-2 decreases neuronal sensitivity to NMDA receptor mediated toxicity, and possibly enhances sensitivity to AMPA receptor mediated toxicity, although we cannot exclude the possibility that FGF-2 treatment sensitizes the neurons to normal system x_C^- mediated glutamate release.

INTRODUCTION

Fibroblast growth factor 2 (FGF-2), despite its discovery in fibroblasts, is widely expressed throughout the brain (Eckenstein, 1994; Dono, 2003). FGF-2 can activate members of the FGF receptor family leading to activation of several intracellular pathways, including PI3K/Akt and MEK/ERK pathways (Reuss and Bohlen und Halbach, 2003). It has been demonstrated to be involved in development (Ohkubo et al., 2004), adult neurogenesis (Mudò et al., 2009), and tissue repair (Reuss and Bohlen und Halbach, 2003). Dysregulation of FGF-2 signaling has been implicated in acute and chronic neurodegenerative diseases, such as stroke (Alzheimer and Werner, 2002), traumatic brain injury (Mellergård et al., 2012), and Alzheimer's disease (Mark et al., 1997), as well as psychiatric disorders such as schizophrenia (Terwisscha van Scheltinga et al., 2010), stress (Molteni et al., 2001; Frank et al., 2007), addiction (Flores and Stewart, 2000), and major depression (Evans et al., 2004). The use of FGF-2 for treatment for stroke in clinical trials has been attempted but without success (Clark et al., 2000; Bogousslavsky et al., 2002).

System x_C^- is a cystine/glutamate antiporter on the cell membrane (Sato et al., 1995b; Liu et al., 2011). Physiologically, system x_C^- takes up cystine and releases

glutamate in exchange at a 1:1 ratio (Sato et al., 1999). The direction of transport is determined by the high glutamate levels and low cystine levels intracellularly. Once cystine is taken up it is rapidly reduced to cysteine, which can then be used to synthesize protein, or glutathione (GSH), an important endogenous antioxidant in the brain (Meister and Anderson, 1983). GSH and cysteine can both be released into the extracellular space, typically by astrocytes, to regulate the redox state of the brain (Wang and Cynader, 2000; Dringen and Hirrlinger, 2003). Also, the extracellular cysteine is taken up by neurons to synthesize GSH (Wang and Cynader, 2000; Lewerenz et al., 2006; Escartin et al., 2011). The glutamate released by system x_C^- can have multiple effects. Glutamate is the most important excitatory neurotransmitter in the central nervous system. Along with mediating excitatory neurotransmission at synapses, glutamate released from astrocytes is also believed to regulate synaptic activity and plasticity through activating parasynaptic and extrasynaptic receptors (Asztely et al., 1997; Hardingham et al., 2002). However, high extracellular glutamate levels can cause neuronal death through excitotoxicity, typically resulting from high NMDA receptor activation leading to excess calcium influx triggering cell death (Choi et al., 1987).

System x_C^- has been shown to be widely expressed, especially in the central nervous system (Sato et al., 2002; Burdo et al., 2006). In vitro cell culture studies have shown that immature neuronal cells rely on system x_C^- to take up cystine (Murphy et al., 1990). When immature neuronal cell cultures are incubated with high levels of glutamate, glutamate competitively inhibits cystine uptake through system x_C^- and the cells die from oxidative stress (Murphy et al., 1989). This mechanism of neuronal death is called oxidative glutamate toxicity (Schubert and Piasecki, 2001). This type of

glutamate toxicity is distinct from glutamate toxicity in mature neuronal cells, which results from over-stimulation of NMDA receptors leading to excessive calcium influx, and it is called excitotoxicity (Choi, 1987). Also, in mature cell cultures, neurons show low levels of system x_C^- activity, while astrocytes exhibit high levels of system x_C^- activity (Lobner, 2009). This shift of function indicates that as cells mature, they take on more specialized tasks and cooperate as a system (Fellin and Carmignoto, 2004; Stipursky et al., 2011; Suzuki et al., 2011).

The dual actions of system x_C^- give it a unique potential for influencing cell fate. System x_C^- activity can be either beneficial or destructive depending on the cellular properties. If cells are susceptible to oxidative stress, increasing system x_C^- activity should be protective as it increases cystine uptake, which can contribute to increased antioxidative defense. However, if the cells express high levels of glutamatergic receptors, it is likely that they will be sensitive to glutamate induced excitotoxicity. Thus, increasing system x_C^- activity might be destructive by increasing extracellular glutamate, which can potentially over-stimulate extrasynaptic glutamatergic receptors.

In the present study, we used primary mixed neuronal and glial cell cultures derived from prenatal mouse cortex to investigate the effect of 48 hour FGF-2 treatment on neuronal survival/death. We have previously demonstrated that 24 hour FGF-2 treatment upregulated system x_C^- activity on astrocytes, but not neurons or microglia. We demonstrate here that after system x_C^- is upregulated for a prolonged period of time, neuronal death occurs due to system x_C^- mediated excitotoxicity.

MATERIALS AND METHODS

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). Serum was from Atlanta Biologicals (Lawrenceville, GA). FGF-2 was from Alomone Labs (Jerusalem, Israel). ^{14}C-cystine was from PerkinElmer (Waltham, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Neuron-enriched cultures were prepared exactly as above with the addition of 10 μM cytosine arabinoside 48 hours after plating to inhibit glial replication. In these cultures <1% of cells are astrocytes (Dugan et al., 1995; Rush et al., 2010). Astrocyte-enriched cultures were prepared as described for mixed cultures except they are from cortical tissue taken from post-natal day 1-3 mice (Choi et al., 1987; Schwartz and Wilson, 1992; Rush et al., 2010). Cultures were maintained in humidified 5% CO_2 incubators at 37°C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Heath

service Policy on Humane Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Induction of neuronal death

All experiments were performed on cultures 13 days in vitro (DIV). Cultures were exposed to different compounds for 48 hours in media as described for plating except without serum.

Assay of neuronal death

LDH release

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells, in the extracellular fluid 48 hours after the beginning of the insult (Koh and Choi, 1987; Lobner, 2000). Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 μM NMDA, or 100% cell death caused by 20 μM of the calcium ionophore A23187, added 24 hours before the assay. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed. Glial cell death (assessed by trypan blue staining) was not observed in any of the current studies.

MTT assay

Cell survival was quantified by the measurement of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue

formazan product (Lobner, 2000). MTT was added to each well 48 hours after the beginning of the insult to the cells (following removal of media for LDH assay). After 30-minute incubation, the medium was removed, and cells were dissolved in dimethyl sulfoxide. The formation of formazan was measured as the amount of reaction product by absorbance change at a wavelength of 590 nm by using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Levels of formazan formation from cultures exposed to 500 μM NMDA (100% neuronal death) were subtracted from insult formazan levels, and results were normalized to a sham wash.

^{14}C-cystine uptake

System x_C- mediated uptake of radiolabeled cystine was assayed as previously described with modifications (Liu et al., 2009). FGF-2 was added to the serum containing media for the indicated durations. Cultures were then washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025μCi/ml) for 20 min. Following ^{14}C-cystine exposure, cultures were washed with ice cold HEPES buffered saline solution and dissolved in 250 μl sodium dodecyl sulfate (0.1%). An aliquot (200 μl) was removed and added to scintillation fluid for counting. Values were normalized to ^{14}C-cystine uptake in untreated control on the same experimental plate.

Statistical analysis

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

RESULTS

As reported previously, 24 hour FGF-2 treatment of mixed neuronal and glial cultures did not cause any cell death (Fig. 4.1.). However, significant neuronal death was observed after 48 hours of FGF-2 treatment (Fig. 4.1.).

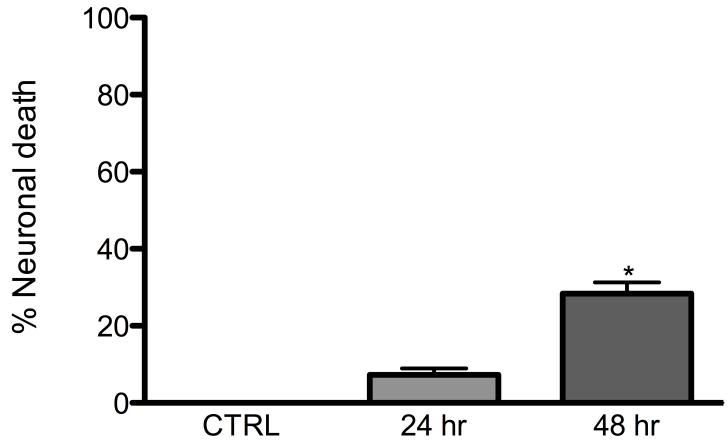

FIGURE 4.1. FGF-2 induced toxicity occurs in mixed neuronal and glial cultures after 48 hour treatment.

Bars show % neuronal cell death (mean ± s.e.m., n = 24) quantified by measuring release of LDH, 24 and 48 hours after the beginning of treatment with 100 ng/ml FGF-2. * indicates significant difference from control.

This neuronal death following FGF-2 treatment for 48 hours is only observed on mixed neuronal and glial cultures, but not neuronal-enriched cell cultures or glial-enriched cell cultures (Fig. 4.2.), suggesting an interaction of glia and neurons may be necessary for FGF-2 induced neuronal death to occur.

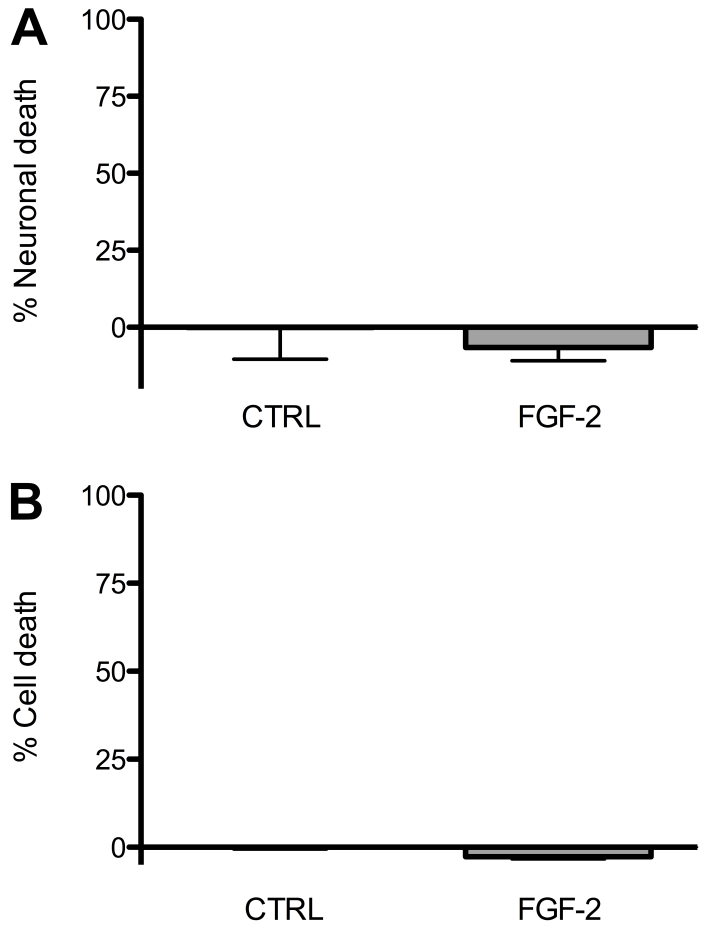

FIGURE 4.2. FGF-2 does not induce toxicity after 48 hour treatment on either neuronal-enriched cultures or astrocyte-enriched cultures.

A: Neuronal-enriched culture. Bars show % neuronal death (mean ± s.e.m., n = 8) quantified by measuring release of LDH, 48 hours after the beginning of treatment with 100 ng/ml FGF-2. B: Astrocyte-enriched culture. Bars show % cell death (mean ± s.e.m., n = 16) quantified by measuring release of LDH, 48 hours after the beginning of treatment with 100 ng/ml FGF-2.

We have shown previously that 24 hour FGF-2 treatment significantly increased system x_C^- activity selectively in astrocytes, with no effect on neuronal-enriched and microglial-enriched cultures (Liu et al., 2011). To determine whether FGF-2 upregulated system x_C^- function is still present after 48 hours we measured 20 min cystine uptake following 48 hour FGF-2 treatment of astrocyte-enriched cultures. FGF-2 treatment

doubled the cystine uptake, and the cystine uptake was mediated by system x_C^- as its inhibitor (s)-4-carboxyphenylglycine (CPG) completely blocked the increase (Fig. 4.3.).

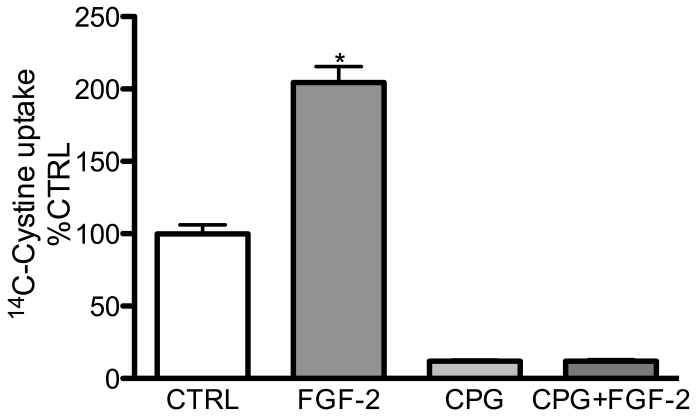

FIGURE 4.3. FGF-2 induced increase in cystine uptake is mediated by system x_C^-. Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 48 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min with or without the system x_C^- antagonist (s)-4-carboxyphenylglycine (200 μM CPG). Bars show % control (mean ± s.e.m., n = 6). * indicates significant difference from control uptake.

To assess whether system x_C^- is involved in the FGF-2 induced neuronal death, we tested the effects of the system x_C^- inhibitors CPG and sulfasalazine (SSZ) on neuronal death induced by 48 hour FGF-2 treatment. Cotreatment of cells with either CPG or SSZ during the 48 hour FGF-2 incubation prevented the neuronal death (Fig. 4.4.). Because SSZ interferes with the LDH release assay, we used the MTT metabolism assay to assess cell survival.

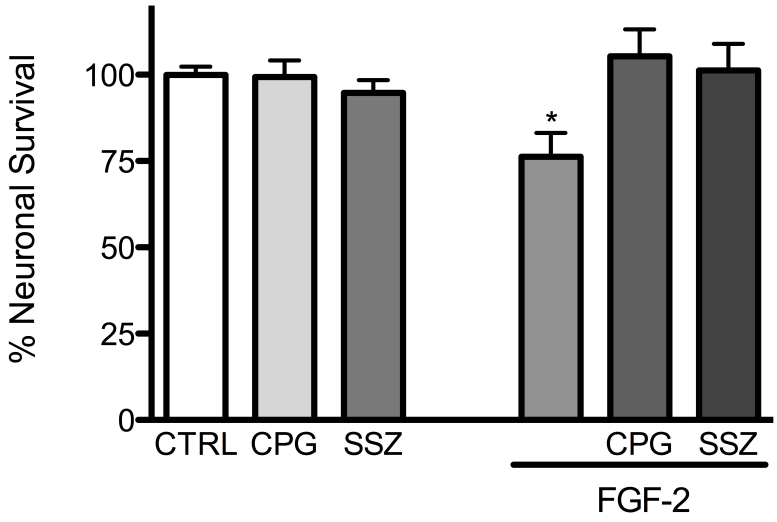

FIGURE 4.4. FGF-2 induced neuronal death is prevented by blocking system x_C^-. Mixed neuronal and astrocyte cultures were exposed to FGF-2 (100 ng/ml) with or without the system x_C^- antagonists (s)-4-carboxyphenylglycine (200 μM CPG) or sulfasalazine (300 μM SSZ) for 48 hours. Bars show % cell survival (mean ± s.e.m., n = 8) quantified by measuring MTT reduction, 48 hours after the beginning of the insult. * indicates significant difference from control.

System x_C^- mediates cystine uptake and glutamate release at the same time: cystine uptake contributes to GSH synthesis that can decrease oxidative stress, therefore, it is not likely to cause cell death; However, increasing glutamate release can lead to over-activation of glutamatergic receptors to cause excitotoxicity. To test this possibility, we used various glutamatergic receptor antagonists to determine if they prevented FGF-2 induced neuronal death. We found that the AMPA receptor antagonist NBQX, but not the NMDA receptor blocker memantine, blocked the FGF-2 induced neuronal death (Fig. 4.5A.). This result is surprising because both NBQX and memantine block 48 hour direct glutamate toxicity (Fig. 4.5B.).

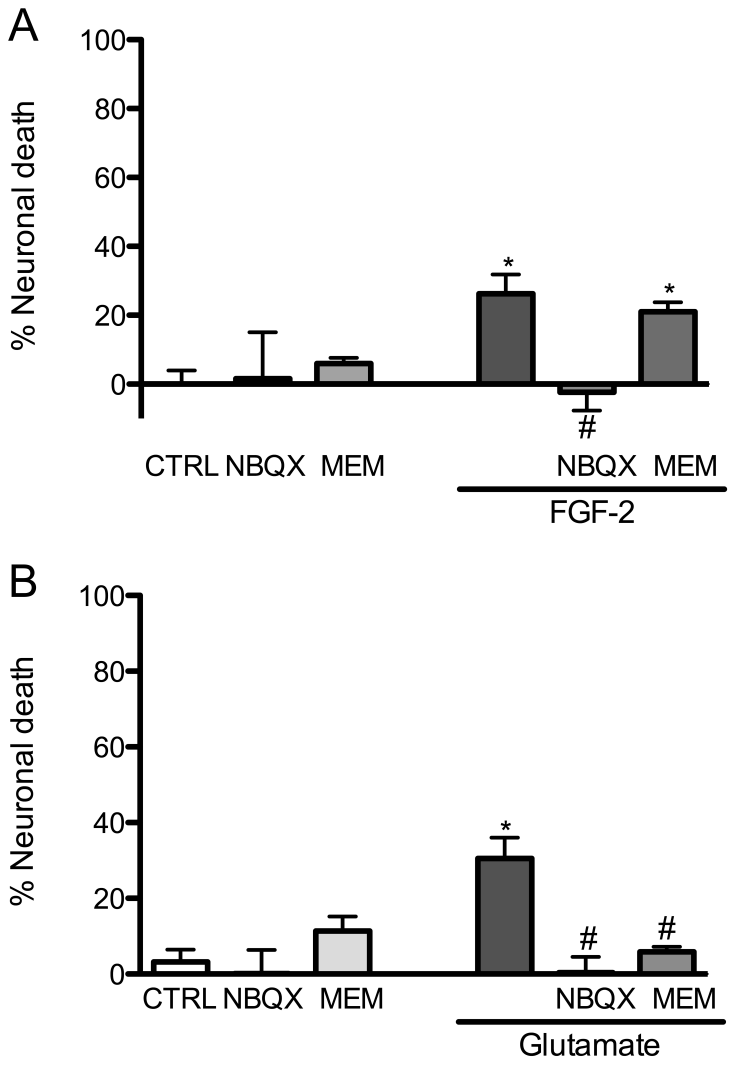

FIGURE 4.5. Effects of the AMPA receptor antagonist NBQX and the NMDA receptor antagonist memantine on neuronal death induced by 48 hour exposure to FGF-2 or glutamate.

A: FGF-2 (100 ng/ml) treatment induced toxicity is protected by blocking AMPA receptors. B: Glutamate (15 μM) induced toxicity is protected by blocking AMPA or NMDA receptors. NBQX: 7.5 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione. MEM: 10 μM memantine. Bars show % neuronal cell death (mean ± s.e.m., n = 12) quantified by measuring release of LDH, 48 hours after the beginning of the insult. * indicates significant difference from control. # indicates significant difference from FGF-2 or glutamate treated.

One possibility for the results is that FGF-2 upregulates calcium permeable AMPA receptors making the neurons susceptible to AMPA receptor mediated toxicity.

However, we did not observe any protection against neuronal death when cultures were cotreated with FGF-2 and various concentrations of the selective calcium permeable AMPA receptor antagonist 1-naphthyl acetyl spermine (NASPM) (Fig. 4.6.).

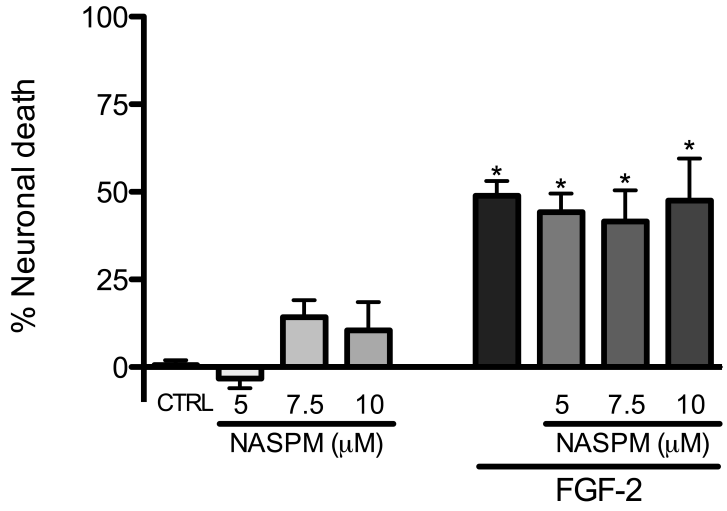

FIGURE 4.6. FGF-2 induced toxicity is not mediated by calcium-permeable AMPA receptors.

Mixed neuronal and glial cultures were exposed to FGF-2 (100 ng/ml) for 48 hours with or without calcium permeable AMPA receptor antagonist 1-naphthyl acetyl spermine (NASPM). Bars show % neuronal cell death (mean ± s.e.m., n = 8) quantified by measuring release of LDH, 48 hours after the beginning of the insult. * indicates significant difference from control.

DISCUSSION

This study examined the effect of FGF-2 on neuronal cell death in mixed neuronal and glial cultures and the involvement of its selective upregulation of system x_C^- on astrocytes. System x_C^- has gained increasingly more attention because of its dual transport property. System x_C^- provides cystine to the cells, which is the rate-limiting source for cysteine, which is one of the three components of glutathione, the major antioxidant in the brain (Dringen et al., 2000; Dringen and Hirrlinger, 2003). In contrast, system x_C^- mediated glutamate release can lead to uncontrolled extracellular glutamate

and cause excitotoxicity (Fogal et al., 2007; Jackman et al., 2010). Both oxidative stress and excitotoxicity are involved in various neurodegenerative diseases and psychiatric disorders. Also, FGF-2 dysregulation is observed in acute and chronic neurological disorders (Gaughran et al., 2006; Terwisscha van Scheltinga et al., 2010; Zechel et al., 2010). Therefore, it is possible that changes in system x_C^- function may be responsible for some of the actions of FGF-2.

Our previous data showed that 24 hour FGF-2 treatment resulted in an increase in cystine uptake through system x_C^- on astrocytes, but with no effect on neuronal and microglial cystine uptake (Liu et al., 2011). FGF-2 treatment mediated increase of system x_C^- was mediated through activation of FGFR1 and required both MEK/ERK and PI3 kinase pathway activations (Liu et al., 2011). All of these cellular events happened without significant neuronal toxicity. However, after 48 hour FGF-2 treatment, significant neuronal cell death began to occur. This neuronal death was blocked by the system x_C^- antagonists CPG and SSZ.

The role of system x_C^- in cell death has been investigated in different cell types and under different conditions. Inhibition of system x_C^- has been shown to be harmful to system x_C^- expressing cells. Oxidative glutamate toxicity was first described on immature neuronal cultures, in which system x_C^- is the major route of cystine uptake (Murphy et al., 1989; 1990). Applying high concentrations of glutamate (millimolar range) to these immature neurons led to GSH depletion and eventually cell death from oxidative stress (Miyamoto et al., 1989; Murphy et al., 1989; Murphy and Baraban, 1990). Under these circumstances, increasing system x_C^- levels would likely be protective.

However, increasing system x_C^- function is not always protective. It seems that increasing system x_C^- function in certain population of cells can be toxic to the surrounding cells. Primary microglia cultured in physiological extracellular concentrations of cystine and glutamine have been shown to be able to release enough glutamate through system x_C^- to kill surrounding neurons (Piani and Fontana, 1994) (Domercq et al., 2007). Activation of these microglia by bacterial components enhanced system x_C^- mediated glutamate release, which was prevented by blocking protein synthesis (Piani and Fontana, 1994). Similarly, the increase in xCT expression in microglia induced by lipopolysaccharide treatment led to increased system x_C^- mediated release of glutamate, causing excitotoxicity to cocultured oligodendrocytes (Domercq et al., 2005). This toxicity was decreased by blocking AMPA receptors with CNQX (Domercq et al., 2005). This result is similar to what we have observed on our primary mixed neuronal and glial cultures. The difference is that oligodendrocytes are normally sensitive to AMPA receptor toxicity (McDonald et al., 1998; Takahashi et al., 2003), while in cortical neurons, glutamate induced excitotoxicity is normally mediated primarily by activation of NMDA receptors (Choi et al., 1987; Choi, 1998).

Similar to microglia, selectively increasing system x_C^- on astrocytes with IL-1β can also lead to increased glutamate release, which potentiates srounding neuronal death induced by hypoglycemia and hypoxia (Jackman et al., 2010; 2012), both of which are well-known to kill neurons in a process that involves over-activation of glutamatergic receptors (Snider et al., 1998; Czyz et al., 2002). The neuronal death in these conditions was largely attenuated by blocking NMDA receptors with the selective NMDA receptor open channel blocker MK-801, and was also blocked by the system x_C^- inhibitor CPG

(Fogal et al., 2007; Jackman et al., 2010; 2012). Our data shows that FGF-2 treatment, like IL-1β, selectively increases system x_C^- activity on astrocytes (Liu et al., 2011). But FGF-2 by itself induced neuronal cell death after system x_C^- activity was upregulated for 48 hours. Also, the pharmacological profile of the excitotoxicity was different from non-FGF-2 treated neurons. We showed that, while neuronal death induced by direct exposure of cultures to glutamate was blocked by either NMDA or AMPA receptor antagonists, FGF-2 induced neuronal death was only attenuated by the AMPA antagonist.

FGF-2 has been shown to be able to change neuronal expression of both NMDA and AMPA receptors, and to alter sensitivity to NMDA receptor mediated toxicity both in vitro and in vivo. A study conducted on cerebellar granule cells reported that FGF-2 treatment caused a time-dependent decrease in expression of NMDA receptor subunits GluN2A and GluN2C with a decrease of NMDA-evoked calcium influx, while GluN1 and GluN2B levels were not changed (Brandoli et al., 1998). Also, FGF-2 pretreatment protected striatal neurons from NMDA receptor mediated toxicity (Freese et al., 1992). Systematic administration of FGF-2 protected against intrastriatal injection of NMDA (Nozaki et al., 1993a; 1993b). FGF-2 treatment enhanced the rate of NMDA receptor inactivation in response to calcium influx in hippocampal neurons (Boxer et al., 1999). FGF-2 treatment decreased NMDA receptor levels in hippocampal cell cultures and elevations in intracellular calcium levels after glutamate exposure compared to non-FGF-2 treated cells (Mattson et al., 1993). FGF-2 treatment has been shown to increase AMPA receptor subunit GluR1 levels in the same cultures, without altering GluR2, GluR3, and GluR4 levels (Cheng et al., 1995). This suggested the possibility that FGF-2 increased the levels of GluR2 subunit lacking AMPA receptors, which have calcium

permeability (Bannerman et al., 2007). However, the selective antagonist of these channels, NASPM, failed to offer protection in our study. At concentrations above 5 μM, NASPM began to induce some toxicity by itself. The IC_{50} of NASPM for calcium-permeable AMPA receptors is 0.33 μM (Brackley et al., 1993; Koike et al., 1997). Therefore, the 5 μM concentration that was not toxic should have been an effective concentration. Therefore, it is possible that the FGF-2 treatment induced increased glutamate release from system x_C- causes an AMPA receptor specific neuronal death because of attenuated NMDA receptor mediated toxicity induced by FGF-2 treatment.

FGF-2 was a potential candidate for the treatment of stroke as it was shown to reduce ischemic damage, as well as, promote recovery and regeneration in multiple in vitro and animal models (Nozaki et al., 1993b; Rosenblatt et al., 1994; Song et al., 2002; Watanabe et al., 2004). However, in human clinical trials, FGF-2 not only failed to show any beneficial effect in treated patients compared to control patients, it actually increased the mortality rate in treated patients (Clark et al., 2000; Bogousslavsky et al., 2002). The reasons for FGF-2 being beneficial in animal models but not in human trials are not yet fully understood. Given that FGF-2 can increase system x_C- activity, and the dual functions of system x_C-, it is not surprising that FGF-2 can have varying effects on cerebral ischemia.

FGF-2 administration has been shown to be beneficial in rodent studies in other disease models, such as depression (Turner et al., 2008c) and Parkinson disease (Hsuan et al., 2006). It is possible that these beneficial effects may be mediated by the FGF-2 effect on system x_C-, which leads to enhanced glutathione synthesis. However, again

taking into account the dual actions of system x_C^-, the effects of long-term treatment with FGF-2 must be rigorously studied.

CONCLUSIONS

The dual amino acid transport function allows system x_C^- regulate intracellular cystine and extracellular glutamate levels at the same time. Cystine taken up is broken down immediately to cysteine, which is a substrate for glutathione synthesis and protein synthesis. While increasing cystine uptake can be protective by increasing the cells' antioxidative defense, glutamate released at the same time can potentially cause toxicity by over activating glutamatergic receptors. We show here that the net effect of long-term upregulation of system x_C^- selectively on astrocytes in mixed neuronal and glial cultures by FGF-2 treatment is negative. That is, the excitotoxicity component dominates, leading to neuronal death.

CHAPTER V

GENERAL DISCUSSION

System x_C^- mediates cystine and glutamate exchange (Sato et al., 1999), thus it is involved in both regulating extracellular glutamate and intracellular GSH due to its dual amino acid transport function. Both abnormal glutamate transmission and chronic oxidative stress have been indicated to contribute to neurodegenerative diseases, such as amyotrophic lateral sclerosis, stroke, traumatic spinal cord/brain injury, Alzheimer's disease and Parkinson's disease (Dugan and Choi, 1994; Andersen, 2004), as well as psychiatric disorders such as addiction (Tsai et al., 1998) and schizophrenia (Pérez-Neri et al., 2006). Therefore, it seems likely that alterations in system x_C^- function may play a role in these diseases. To begin to resolve this question, a series of studies were conducted to find out how system x_C^- is involved in brain cell health in primary cortical cell cultures. We found that the environmental neurotoxin BMAA can compete with cystine at system x_C^- leading to both oxidative stress and excitotoxicity, that FGF-2 can upregulate system x_C^- selectively on astrocytes, and that the upregulation of system x_C^- by FGF-2 treatment may be responsible for AMPA receptor specific neuronal death caused by 48 hour treatmentFGF-2 . Following is a discussion of some of the issues that arose during these studies.

INTERACTION OF FGF-2 AND BMAA ON NEURONAL DEATH

Since both BMAA and FGF-2 have actions on system x_C^-: FGF-2 upregulates system x_C^- function (Liu et al., 2011) and BMAA competes with cystine at system x_C^- (Liu et al., 2009), we examined whether FGF-2 pretreatment alters BMAA toxicity. FGF-2 pretreatment for 24 hours changed the concentration response of BMAA toxicity (Fig. 5.1). At low concentrations, BMAA toxicity was potentiated by FGF-2

pretreatment, while at high concentrations, FGF-2 pretreatment protected neurons from BMAA toxicity.

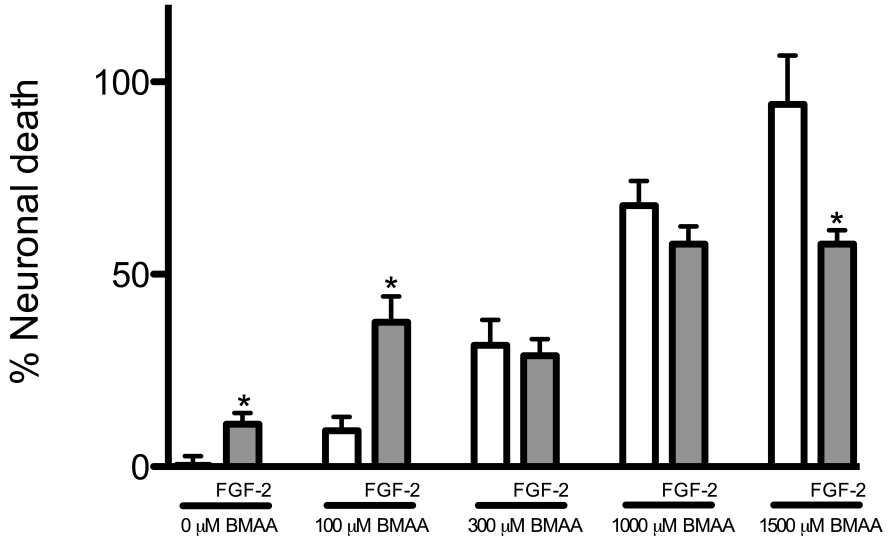

FIGURE 5.1. Concentration response of BMAA induced neuronal death after FGF-2 pretreatment.

Mixed neuronal and glial cultures were pretreated with 100 ng/ml FGF-2 for 24 hours before various concentrations of BMAA were added. Media was sampled for LDH assay 24 hours after the beginning of the BMAA insults. Bars show % neuronal cell death (mean ± s.e.m., n= 8) quantified by measuring release of LDH. * indicates significant difference from control (no BMAA).

The mechanism to explain this result may be very complicated, since both BMAA and FGF-2 have multiple effects on cells. BMAA can decrease glutathione by decreasing cystine uptake (Liu et al., 2009), inhibiting glutathione synthesis (Esterhuizen-Londt et al., 2011), and potentially be incorporated into glutathione. With bicarbonate present, BMAA mimics glutamate and directly activates glutamatergic receptors (Weiss and Choi, 1988; Rao et al., 2006; Lobner et al., 2007). FGF-2, as a growth factor, has a wide range of effects on both neurons and glia (Beck, 1994; Eckenstein, 1994; Ghosh and Greenberg, 1995; Temple and Qian, 1995; Grothe and Wewetzer, 1996; Ford-Perriss et al., 2001; Reuss and Bohlen und Halbach, 2003; Mudò et al., 2009).

If we consider system x_C^- as the main mediator of the effects of both compounds, the most likely explanation for the data is as follows. At low levels of BMAA, when system x_C^- is upregulated by FGF-2 pretreatment, BMAA drives increased glutamate release from system x_C^-, which exacerbates BMAA toxicity by over activating glutamatergic receptors. The result could also be explained by a different mechanism: FGF-2 pretreatment may potentiate low level BMAA toxicity by upregulating system x_C^- allowing more BMAA to enter the cells and through inhibition of enzymes or incorporation into glutathione act to decrease cellular glutathione levels. However, at high levels of BMAA, excitotoxicity caused by direct activation of glutamatergic receptors by BMAA is already maximized, and no further excitotoxicity can be induced, but elevated system x_C^- function by FGF-2 pretreatment provides cells with more cystine which strengthens antioxidative defense, thus providing protection against BMAA toxicity.

Due to the complicated actions of both BMAA and FGF-2, and the lack of direct scientific implication of their interaction occurring, no further studies were performed concerning BMAA and FGF-2 interactions.

CONTROVERSIES REGARDING BMAA

In chapter two of this thesis we examined the mechanisms of BMAA toxicity and found that one novel way in which it kills neurons is through actions on system x_C^-. It not only competitively inhibits cystine uptake leading to glutathione depletion, but also increases glutamate release, causing excitotoxicity. This is potentially a very important result because it provides a novel mechanism for BMAA toxicity, but it is only important

if BMAA does play a role in neurodegenerative diseases. In this section, we will explore some of the controversies regarding the potential role of BMAA in neurodegenerative diseases.

Is BMAA a potent neurotoxin?

One major question concerning BMAA is whether it can cause neuronal death at low enough concentrations to make it a potential factor in neurodegenerative diseases. Studies in cortical cell culture have shown that very high BMAA concentrations (1 - 3 mM) are required to induce neuronal death (Ross et al., 1987; Weiss and Choi, 1988; Weiss et al., 1989b; Lobner et al., 2007). One potential mechanism by which BMAA may be involved in neurodegenerative diseases is that it is not acting as the sole cause of the disease, but may act in concert with other environmental or genetic factors. In support of this possibility it was found that BMAA, at concentrations as low as 10 μM, potentiated amyloid-β and 1-methyl-4-phenylpyridinium ion (MPP+) toxicity, models of Alzheimer's and Parkinson's disease, respectively (Lobner et al., 2007).

We also looked into the possibility of BMAA causing selective toxicity of specific vulnerable populations of neurons. Mesencephalic and septal primary cultures were prepared to study the death of dopaminergic neurons and cholinergic neurons, which are associated with Parkinson's disease and Alzheimer's disease, respectively. The small percentage (0.1%-1%) of dopaminergic and cholinergic neurons present in the two cultures allowed for the analysis of death of these specific cell populations at the same time as death of the total neuronal population. That is, the total neuronal death was assessed by the LDH release assay, and then the cells were fixed and stained, with the

number of dopaminergic or cholinergic cells counted. In mesencephalic cultures there was no difference between the sensitivity of the total neuronal population and the dopaminergic neurons to BMAA toxicity (Fig. 5.2A). In contrast, in the septal cultures, the cholinergic neurons were significantly more sensitive than the total neuronal population at both the 30 and 300 μM BMAA concentrations (Fig. 5.2B). The LC_{50} values calculated by nonlinear regression analysis were: dopaminergic neurons – 304 μM; total mesencephalic neurons – 241 μM; cholinergic neurons – 32 μM; total septal neurons – 586 μM.

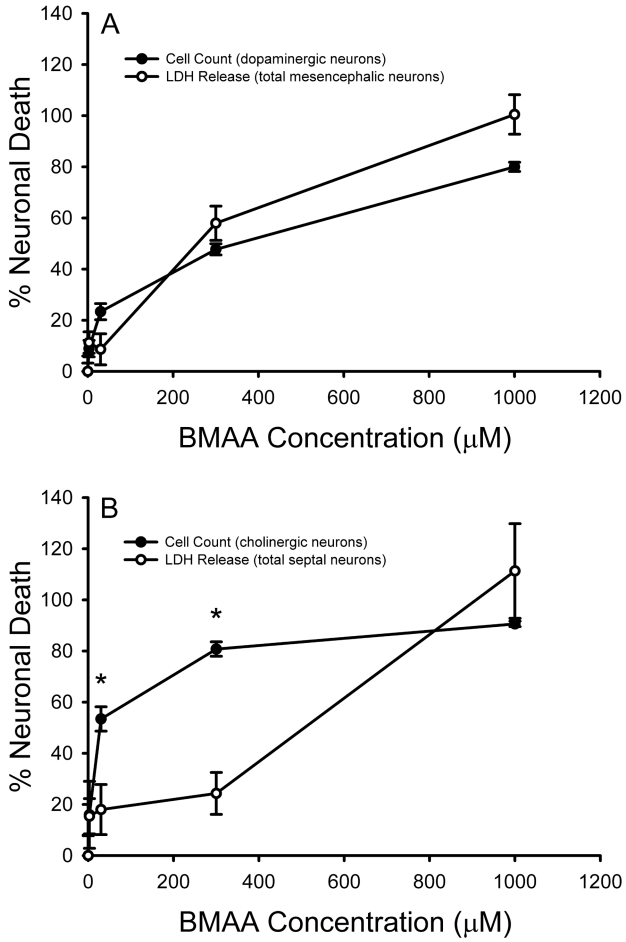

FIGURE 5.2. Cholinergic, but not dopaminergic neurons are selectively sensitive to BMAA toxicity.

BMAA concentrations of 3 μM, 30 μM, 300 μM, and 1 mM were present for 24 hours. The cell counting data shows % dopaminergic (A) or cholinergic (B) neuronal death (mean ± SEM, n = 8) quantified by counting the number of TH or ChAT-positive neurons. The LDH release data shows % total neuronal cell death (mean ± SEM, n = 8). * indicates significant difference between the LDH release and cell counting data.

To determine the mechanism of BMAA toxicity in septal cultures we assayed the protective effects of the NMDA receptor antagonist MK-801, the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), and the free radical scavenger trolox on both total neural death and specific cholinergic neuronal death. To match the levels of death, 500 μM BMAA was used for total septal neuronal death, and 300 μM BMAA for cholinergic neuronal death. For total septal

neuronal death, protection was provided by MK-801 and trolox, but not NBQX (Fig. 5.3A.). This is consistent with our previous results in cortical cultures where we found evidence for both NMDA receptor and free radical mediated neuronal death (Lobner et al., 2007). In contrast, the death of cholinergic neurons was not attenuated by MK-801 or trolox, but was decreased by NBQX (Fig. 5.3B.).

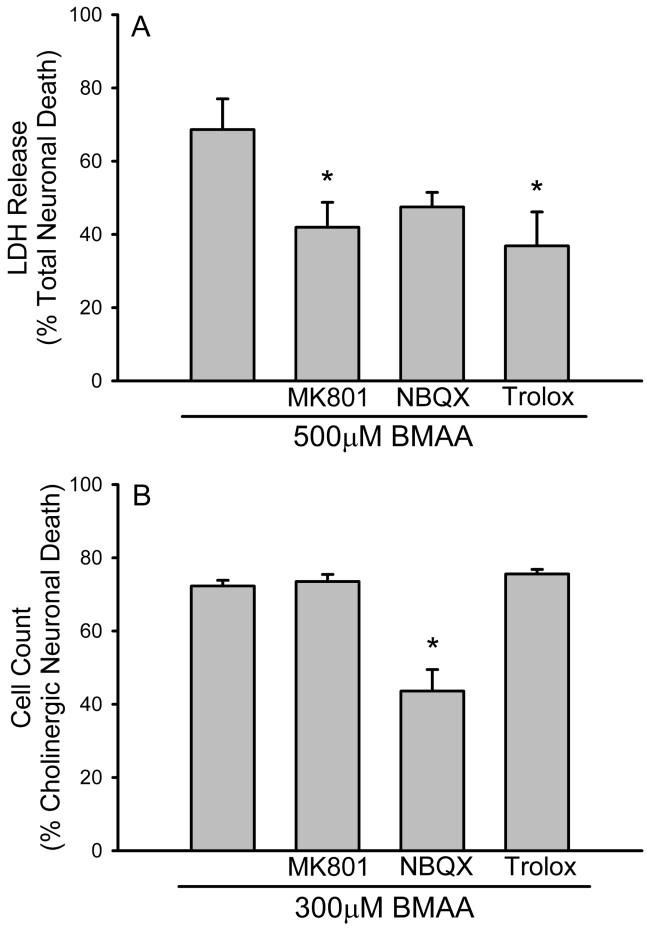

FIGURE 5.3. MK801 (10 μM) and trolox (100 μM) provide protection against BMAA toxicity to total septal neurons (A), while NBQX (20 μM) provides protection of cholinergic neurons (B).

Bars show % neuronal cell death (mean + SEM, n= 12) quantified by measuring release of LDH (A) or counting of cholinergic neurons (B). * indicates significant difference from control.

In cortical cultures, where at least 1mM BMAA is required to induce general neuronal death (Ross et al., 1987; Weiss and Choi, 1988; Weiss et al., 1989b; Lobner et al., 2007). However, death of the small subpopulation of NADPH-diaphorase cells occurs at a BMAA concentration of 100 μM, because these NADPH-diaphorase cells are characterized by high levels of calcium permeable AMPA receptors (Weiss et al., 1989b). Similarly, motor neurons, which are also characterized by calcium permeable AMPA receptors, have also been shown to be highly sensitive to BMAA toxicity, with death occurring at a concentration of 30 μM (Rao et al., 2006). Furthermore, like NADPH-diaphorase cells, this death is mediated by activation of AMPA/kainate receptors (Weiss et al., 1989a; Carriedo et al., 1995). Cholinergic neurons have also been shown to possess high levels of calcium permeable AMPA receptors (Yin et al., 1994). The results indicated that neurons containing high levels of calcium permeable AMPA receptors are more sensitive to BMAA toxicity. However, the protective effect of NBQX was not complete, suggesting that other mechanisms of toxicity are also involved.

Rapid electrophysiological studies have established that the effects of BMAA on NMDA receptors are due to direct actions on the receptor (Weiss et al., 1989b; Lobner et al., 2007). BMAA has been shown to bind to AMPA receptors (Rakonczay et al., 1991; Cucchiaroni et al., 2010), and cause convulsions when injected into the lateral ventricle of rats that are blocked by AMPA antagonists (Matsuoka et al., 1993). In our lab, we found that BMAA is a substrate for system x_C-, and through this action drives glutamate release (Liu et al., 2009). Therefore, an alternative explanation for BMAA being a direct AMPA receptor agonist is that BMAA may stimulate the release of endogenous glutamate, which activates the glutamatergic receptors. In this case, the presence or

absence of calcium permeable AMPA receptors would determine the sensitivity of the cells to the toxicity of the endogenous glutamate.

The importance of these results is that they show that specific, and functionally important, populations of neurons are sensitive to BMAA toxicity at concentrations that are much lower than the general cortical neuronal population, making it more likely that BMAA plays a role in neurodegenerative diseases where cell death of specific populations occurs.

Measurement of BMAA

Another area of controversy is the detection and quantification of BMAA in tissues. There are labs that have successfully measured and quantified it in multiple types of tissues (Cox and Sacks, 2002; Murch et al., 2004a; Pablo et al., 2009), while there are also labs that have failed to detect BMAA in similar systems (Montine et al., 2005; Snyder et al., 2009). Different methods used in these studies may be responsible for the lack of detection (Crimmins and Cherian, 1997). A recent review by Steve Cohen of Waters Corp, the developer of multiple amino acid detection methods, suggested that using HILIC columns with tandem mass spectrometry, the method used by all of the investigators not detecting BMAA, may not be detecting BMAA because of the high level of ionization suppression due to much higher concentrations of other matrix components (Cohen, 2012). Another complication is that it has been shown that the majority of BMAA in tissue is protein bound (Murch et al., 2004b). This may be occurring through misincorporation into cellular proteins by BMAA being mischarged to the tRNAs (unpublished data). Therefore, the processing method of samples becomes

important in whether BMAA is detected (Crimmins and Cherian, 1997; Murch et al., 2004b).

BMAA in animal models

Several studies have been conducted to examine BMAA effects in vivo. Macaques were fed 100-350 mg/kg BMAA daily for 13 weeks, after which corticomotoneuronal dysfunction, Parkinsonian features and behavioral abnormalities were observed (Spencer et al., 1987). However, the study was criticized for the high levels of BMAA that was tested (Duncan et al., 1990). Intracerebroventricular administration of 0.5 mg/day of BMAA to rats up to 60 days old caused cholinergic cell degeneration as early as 16 days after BMAA administration and general cortical neuronal damage after 40 days (Rakonczay et al., 1991). With the same BMAA administration, behavioral changes such as splay, jerking movements, and rigidity were elicited 6 days after administration (Rakonczay et al., 1991; Matsuoka et al., 1993). Although the symptoms elicited by BMAA administration are not exactly the same as observed in ALS-PDC, considering the difficulty in modeling the effects of chronic BMAA exposure, it was encouraging to observe neuronal toxicity and behavior changes following BMAA administration. However, these studies involve intracerebroventricular injection, while exposure in humans is through ingestion. There are a number of studies that failed to observe any behavioral responses following oral intake of BMAA. Force-feeding a total of 15.5 g/kg (500mg/kg or 1000 mg/kg doses) of BMAA to Cynomolgus monkeys for 11 weeks did not induce any behavior changes, nor any neurotoxicity in the cerebral cortex (Perry et al., 1989). In another study, feeding adult mice 28 mg/kg of

BMAA per day for 30 days caused no motor, cognitive or neuropathological changes (Cruz-Aguado et al., 2006). Therefore, whether chronic BMAA administration induces any behavioral or neuropathological changes is still controversial and requires more study. One problem is that humans may be exposed to BMAA for decades, which cannot be mimicked in animal models. Also, animal studies have involved administration of BMAA to young, healthy animals, which does not mimic the complex changes occurring in the aging human brain, or interactions with other environmental and genetic factors.

Is action on system x_C^- an important mechanism of BMAA toxicity?

We showed that along with the already known action of BMAA of activating glutamatergic receptors, BMAA also acts as a substrate for system x_C^- decreasing cystine uptake and increasing glutamate release. Thus, by acting at system x_C^-, BMAA can cause both oxidative stress and excitotoxicity, both of which occur during neurodegenerative diseases. This makes it an attractive mechanism to explain BMAA's potential role in neurodegenerative diseases. BMAA producing cyanobacteria exist ubiquitously, and it can get into the food supply, by accumulation in fish, crabs, and oysters (Banack et al., 2007; Cox et al., 2009; Brand et al., 2010; Jonasson et al., 2010; Li et al., 2010). Therefore, it is important to study the potential threats of BMAA and understand the role of system x_C^- in its mechanism of toxicity.

REGULATION OF SYSTEM XC- BY FGF-2

In chapter three of this thesis, we presented data showing the novel action of FGF-2 on astrocytes: increasing system x_C^- function (Liu et al., 2011). This effect was

mediated by FGFR1, and required activation of both the PI3K/Akt and MEK/ERK pathways. It has been well accepted that FGF-2 is involved in many aspects of central nervous system function and regulation (Eckenstein, 1994; Dono, 2003; Reuss and Bohlen und Halbach, 2003). This new action of FGF-2 might provide insights into some of its effects in the brain.

FGF-2 in the central nervous system

FGF-2 is a well studied growth factor that has been shown to be more prevalent in the central nervous system than the peripheral organs (Ledoux et al., 1992). By interacting with four cell surface tyrosine kinase FGF receptors and acting through several intracellular pathways, FGF-2 has been shown to be involved in development (Ohkubo et al., 2004), adult neurogenesis (Mudò et al., 2009), and tissue repair (Reuss and Bohlen und Halbach, 2003). FGF-2 may be involved in acute and chronic neurodegenerative diseases such as stroke (Alzheimer and Werner, 2002) and Alzheimer's disease (Mark et al., 1997). FGF-2 expression can be changed. Processes such as injury, stress, seizures, learning and memory all have been shown to increase FGF-2 expression (Dono, 2003; Fumagalli et al., 2005). FGF-2 dysregulation has been suggested in schizophrenia (Terwisscha van Scheltinga et al., 2010), stress (Molteni et al., 2001; Frank et al., 2007), addiction (Flores and Stewart, 2000), major depression (Evans et al., 2004), as well as traumatic brain injury (Mellergård et al., 2012). Its important role in neurogenesis and tissue repair in adult makes FGF-2 an attractive candidate for treatment of various neurodegenerative disorders.

FGF-2 and oxidative stress

There is substantial evidence indicating that system x_C^- is induced when cells suffer from oxidative stress or low GSH (Sato et al., 2004; Seib et al., 2011; Lewerenz et al., 2012; Bridges et al., 2012b). The effects of FGF-2 on oxidative stress are unclear. It has been shown that FGF-2 potentiates oxidative stress mediated neuronal death (Lobner et al., 2003). However, FGF-2 has also been shown to be protective against oxidative stress mediated toxicity (Yang and de Bono, 1997; Yamada et al., 2001). Regarding the possibility that the FGF-2 upregulation of system x_C^- that we observed was mediated by oxidative stress, co-treatment of the free radical scavenger trolox with FGF-2 did not prevent the increased system x_C^- mediated cystine uptake induced by FGF-2 treatment (Fig. 5.4.). We have shown previously that trolox blocks methylmercury induced increased system x_C^- activity, which we believe was mediated by decreased glutathione/oxidative increased stress (Rush et al., 2012b). Therefore, it is unlikely that the FGF-2 treatment induced increase in system x_C^- function was mediated by oxidative stress.

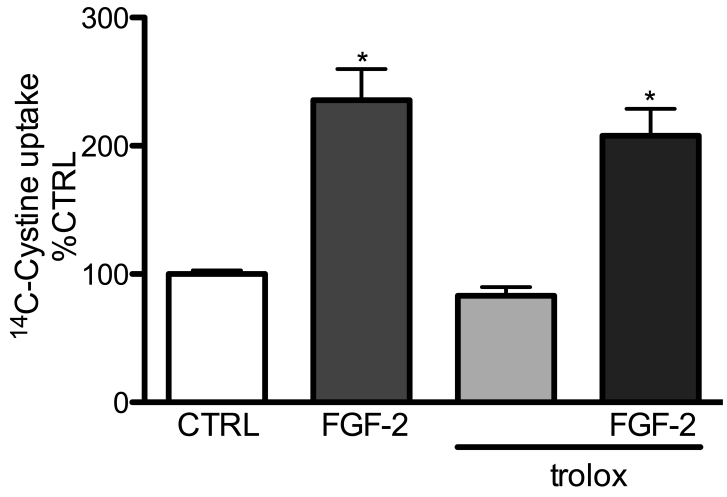

FIGURE 5.4. FGF-2 induced increase in cystine uptake is not oxidative stress dependent.

Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) with or without the free radical scavenger trolox (100 μM) for 24 hours, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min. Bars show % control (mean ± s.e.m., n = 6). * indicates significantly different from control uptake.

FGF-2 and glutamate

It is well accepted that there is an interaction between FGF-2 and glutamate neurotransmission. FGF-2 is required for normal generation of glutamatergic projections in vivo (Korada et al., 2002). Decreased glutamatergic neurons and glutamatergic transmissions were reported in FGF-2 deficient mice (Fadda et al., 2007). Mice carrying a mutation in fgfr1 gene showed decreased glutamatergic transmission in frontal and temporal cortex (Shin et al., 2004). FGF-2 also promotes glial expression of glutamate transporters (Figiel et al., 2003), and reduced NMDA receptor activity in vivo (Fumagalli et al., 2004).

We also investigated whether glutamate has an effect on system x_C^- activity. We used 50 μM bicuculline, a GABA(A) receptor blocker, and 2.5 μM 4-AP, a potassium channel blocker, to cause the presynaptic release of neurotransmitters and selectively

activate synaptic receptors. In our primary cortical cultures, the majority of the synapses are glutamatergic synapses. Our preliminary data suggested that synaptic glutamate release can lead to increased cystine uptake within 20 min (Fig. 5.5.), and the cystine uptake was mediated through system x_C^- because it was CPG sensitive (Fig. 5.5.). The effect, while significant, was small and variable among different plates. When we continued these studies by testing the effect of various glutamatergic receptor antagonists on the effect of bicuculline/4-AP of increasing cystine uptake, the increase in cystine uptake disappeared. Also, we were unable to determine whether the increase of system x_C^- function is caused by synaptic glutamate release. Therefore, this line of study was not pursued further.

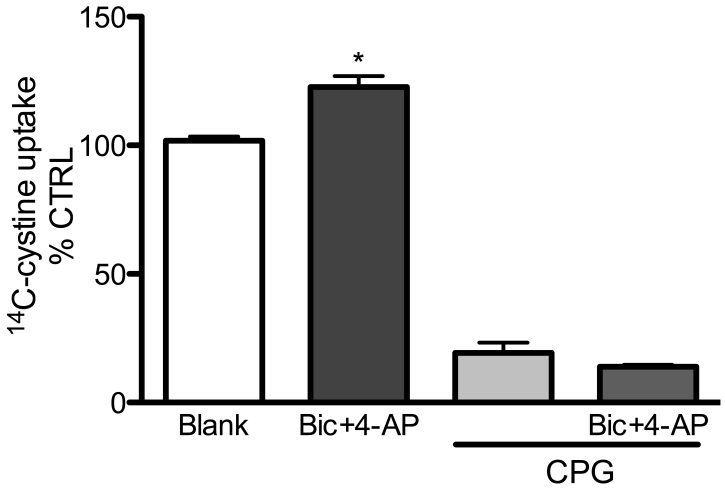

FIGURE 5.5. Short-term synaptic glutamate release increases system x_C^- activity. Mixed neuronal and glial cultures were exposed to (-)bicuculline methochloride (Bic, 50 μM) and 4-aminopyridine (4-AP, 2.5 μM) for 20 min, then ^{14}C-cystine is added to the media, and ^{14}C-cystine uptake is measured for 20 min. Bar show % control (mean±s.e.m., n=24). * indicates significantly different from control uptake.

TOXICITY OF FGF-2 MEDIATED BY SYSTEM XC-

Despite the numerous reports of FGF-2 as a neuroprotective growth factor (Alzheimer and Werner, 2002), cytotoxic effects of FGF-2 have also been reported. FGF-2 inhibits growth of cultured breast cancer cells (Johnson et al., 1998; Wang et al., 1998), Ewing's sarcoma cells (Schweigerer et al., 1987), and human neuroblastoma cells (Russo et al., 2004). In Ewing's sarcoma tumors, the cytotoxic effect of FGF-2 is mediated through the same pathways that were associated with FGF-2 induced cell proliferation (Kim et al., 2004; Russo et al., 2004; Williamson et al., 2004; Ma et al., 2008). 48 hour treatment of FGF-2 inhibits Ewing's sarcoma growth by inducing cell death in three different cell lines (Sturla et al., 2000; Ma et al., 2008). FGF-2 also inhibits Ewing's sarcoma growth in vivo (Sturla et al., 2000). Overexpressing FGF-2 in mice reduces chondrocyte proliferation by increasing apoptosis (Sahni et al., 2001).

We also observed significant neuronal death 48 hours after FGF-2 treatment, which was associated with upregulation of system x_C- activity. Blocking either system x_C- or AMPA receptors eliminated the neuronal cell death, suggesting glutamate exported from astrocytic system x_C- contributes to the neuronal death. However, we cannot exclude the possibility that the neuronal toxicity was due to FGF-2 sensitizing the neurons to normal glutamate release from system x_C-. An AMPA receptor antagonist, NBQX, but not an NMDA receptor antagonist, MEM, blocked the FGF-2 induced neuronal death. This profile of toxicity is different from non-FGF-2 treated cells, in which low level glutamate exposure causes a similar level of neuronal death, but it is prevented by either AMPA or NMDA receptor antagonists. We could not use the more potent NMDA receptor antagonists (2R)-amino-5-phosphonovaleric acid (APV) or MK-

801 because they cause cell death by themselves, even at low concentrations, after 48 hours (data not shown). Bath incubation of glutamate is believed to activate extracellular receptors, which is likely similar to glutamate released from system x_C-. Therefore, FGF-2 is in some way altering the type of excitotoxicity that occurs to the neurons. To determine what FGF-2 changes in terms of response to glutamate, detailed studies would need to be carried out examining whether the levels of NMDA and AMPA receptors were changed. This could be done by western blotting, examining the levels of NMDA and AMPA receptor subunits. Furthermore, electrophysiological studies would be needed to examine whether functional changes in receptors occurred. Fura-2 studies could also be carried out to examine whether calcium influx was changed by FGF-2 treatment. It is also possible that the changes in glutamate receptor mediated toxicity are due to changes in the intracellular signaling cascade downstream of receptor activation.

We made several attempts to measure whether there was elevated glutamate accumulating in the culture media following 48 hour FGF-2 treatment. DIV 13 astrocyte-enriched cultures were washed into fresh media, then following 48 hour exposure to FGF-2 or not (CTRL), bathing media from the cell cultures was collected and assayed for glutamate by HPLC. The media used for such long-term exposure was requred to be a complex media we call MS, which contains more than 100 μM concentrations of 8 amino acids including cystine. Astrocyte-enriched cultures were used because in mixed cultures neuronal death would occur at this time point, and the dead neurons would have released large amounts of glutamate. Since the FGF-2 effect on system x_C- occurs on astrocytes, the effect should be observed in the astrocyte-enriched cultures. Two HPLC methods were tried to quantitate glutamate: phenylisothiocyanate (PITC) derivatization with

ultraviolet detection on an Agilent 1100 HPLC and fluorescence detection with ophthaldehyde pre-column derivitization on a Shimadzu LC10AD HPLC. However, because the experiments were done in a complex media, there was an interfering peak where glutamate was to be measured. Both a Hypersil-ODS reverse phase column and a Synergi Hydro-RP column were tested with the PITC derivatization with ultraviolet detection method in an attempt to separate the interfering peak from the glutamate peak. Also various mobile phases and flow rates were tested. Although the new column, and using a slower flow rate appeared to give us better separation, it significantly decreased the sensitivity for detection of glutamate (more than 10 times less sensitive). Since we are detecting glutamate in a large amount of extracellular media compared to that of the restricted space among the attached cells (with an estimated 1:1000 dilution), the glutamate concentration in the extracellular media is very low. Furthermore, it is not known whether FGF-2 treatment changes the glutamate uptake efficiency. Therefore, we were unable to determine the extracellular glutamate levels under these conditions.

We cannot exclude the possibility of FGF-2 treatment changing neuronal susceptibility to normal extracellular glutamate levels. One of the possibilities being that FGF-2 treatment changes the astrocytes' ability to release cysteine-containing molecules, which in turn, selectively decreases glutathione levels in neurons, causing them to be susceptible to any insult, including glutamate levels that does not cause death to healthy neurons. This hypothesis could be tested by measuring thiols by HPLC in the culture media of mixed neuronal and glial cultures.

IS SYSTEM XC- ACTIVITY TOXIC TO NEURONS?

In this dissertation, we have shown that BMAA can contribute to neuronal death by acting at system x_C-. Also, that prolonged FGF-2 treatment can upregulate system x_C- and cause a system x_C- function dependent neuronal toxicity. As knowledge of the function of system x_C- expands, and the interest in targeting this system as a treatment for various neurodegenerative diseases and psychiatric disorders increases, it is important to understand the potential damage that system x_C- activity can cause. Currently, it is clear that for cells that mainly rely on system x_C- to take up cystine for the production of glutathione, blocking system x_C- function can be toxic (Murphy et al., 1989). The question remains whether the glutamate exchanged out of these cells by system x_C- is a threat to surrounding cells. Glioma cells have increased system x_C- function, as well as decreased glutamate uptake, leading to increased extracellular glutamate which can cause toxicity to the surrounding neurons (Ye and Sontheimer, 1999; Sontheimer, 2003; 2008; Ogunrinu and Sontheimer, 2010). Both FGF-2 (Liu et al., 2011) and IL-1β (Fogal et al., 2007) upregulate system x_C- function selectively on astrocytes. Long-term FGF-2 treatment causes a system x_C- function dependent, and AMPA receptor specific, neuronal death. While IL-1β treatment potentiates hypoxia and glucose deprivation induced neuronal death through a system x_C- activity dependent mechanism (Fogal et al., 2007; Jackman et al., 2012). Another example of increased system x_C- function is activated microglia, which release enough glutamate through system x_C- to cause toxicity to the surrounding neurons (Domercq et al., 2007). In all of these studies, along with the involvement of system x_C-, the neurons are under stress: hypoxia, glucose deprivation, or stimulation by growth factors or cytokines. Therefore, while it is clear that enhanced

system x_C- activity on non-neuronal cells can potentiate neuronal death when the neurons are compromised, it is uncertain whether glutamate release from system x_C- can cause neuronal death under normal conditions.

QUESTIONS REGARDING SYSTEM XC- STUDIES

Molecular properties of system x_C-

Northern blot has shown three different xCT transcripts (12, 3.5 and 2.5 kb) in cultured macrophages and HT22 cells, as well as in various mouse organ tissues (Sato et al., 1999; 2001; 2004; 2005; Lewerenz et al., 2006; Taguchi et al., 2007). They may represent alternative splicing or alternative polyadenylation (Sato et al., 1999; Lewerenz et al., 2006). While all of these transcripts are inducible to a similar extent in culture (Sato et al., 1999), they have a different distribution pattern. The 3.5 and 2.5 kb transcripts were predominantly seen in macrophages, while the 12 kb RNA transcript was found in mouse brain, but not in peripheral organs such as heart, lung, liver, and kidney (Sato et al., 1999; Bassi et al., 2001; Sato et al., 2002). Human ovarian cancer cell lines (Okuno et al., 2003), hamster kidney cells (Sasaki et al., 2002), and cultured rat astrocytes (Gochenauer and Robinson, 2001)were shown to exclusively express the 12 kb transcript of xCT.

The predicted amino acid sequence from cDNA for xCT would be a protein of 502 amino acids with a relative molecular mass of 55.5 kDa (Sato et al., 1999). However, in reality, xCT with various molecular masses have been reported. The 55kDa protein is observed both in brain in vivo and in cortical neuronal stem cell cultures (Sims et al., 2012). 50 kDa xCT was detected in human glioma cells (Kim et al., 2001) and

retinal ganglion cell line membranes (Dun et al., 2006). In vitro expression of xCT in Xenopus oocytes (Sato et al., 1999) and HT22 cells (Burdo et al., 2006) showed a band of an approximately 40 kDa protein, which could be downregulated by siRNA (Burdo et al., 2006). This 40 kDa xCT was detected in rat brain tissue, with the expression level increasing during development and reaching the highest expression as an adult (La Bella et al., 2007). xCT was also observed in mouse hippocampus at a molecular weight of ~35 kDa, which is absent in xCT -/- mice (Pacchioni et al., 2007; De Bundel et al., 2011).

There are also studies reporting that xCT is detected at multiple molecular masses in the same tissue, with the same antibody. A study done in primary rat astrocyte cultures revealed three distinct bands for xCT: 40, 45, and 50 kDa, however, only the 40 kDa was released from the system x_C^- heterodimer after reducing agent was added (Seib et al., 2011). xCT was also detected at 50 kDa and 40 kDa in mouse Muller cells, with the 50 kDa protein as the majority version. However, it was not inducible by oxidative stress, while the minority 40 kDa form, which is normally predominantly located intracellularly, was upregulated by oxidative stress and translocated to the membrane (Mysona et al., 2009). The same 40 kDa form of xCT was also found to be almost exclusively present on the cellular membrane in rat brain tissue, cultured human fibroblast, rat neurons and astrocytes (La Bella et al., 2007). xCT was detected at 35 kDa and 55 kDa in mouse brain and mouse astrocyte cultures, with the 55 kDa form prominently detected only in mouse meninges (Shih et al., 2006).

Together with these mixed results, there are also a number of system x_C^- studies that did not report the band(s) mass in their paper (Bridges et al., 2004; Qiang et al., 2004; Sakakura et al., 2007). It seems that the use of different antibodies and different

tissues can lead to different results. Furthermore, increased expression does not necessarily mean increased membrane expression and increased function. Therefore, claims about potential system x_C^- function by measuring xCT protein expression must be considered skeptically. In our studies, we have focused on the functional activity of system x_C^- measured by ^{14}C-cystine uptake.

Relevance and limitations of translating in vitro studies to in vivo:

It has been suggested that when compared to in vivo, system x_C^- activity is much higher in vitro (Lewerenz et al., 2012). This is believed to be because system x_C^- activity can be easily induced. Even regular cell culture conditions with ambient O_2 of 21% strongly induces system x_C^- activity in diverse cell types, including fibroblasts, HEK cells, hippocampal HT22 cells, astrocytes, and microglial (Lewerenz et al., 2012). In freshly prepared hepatocytes and macrophages, the activity of system x_C^- is barely detectable (Takada and Bannai, 1984; Watanabe and Bannai, 1987), but it is significantly upregulated after 12 hours of culturing (Watanabe and Bannai, 1987). A much higher system x_C^- activity level is measured in macrophages cultured for 8 hours in the presence of LPS and diethyl maleate (Sato et al., 1995a). Various electrophilic agents and different pH have been proven to be system x_C^- activity inducers (Reynolds et al., 1991; Sasaki et al., 2002). These concerns regarding cell culture studies must be kept in mind when attempting to extrapolate cell culture studies to the in vivo situation.

Our in vitro data showed that astrocytes have greater system x_C^- activity than neurons (Lobner, 2009), in agreement with another lab (Jackman et al., 2012). Preliminary data from our lab indicates that neuronal conditioned media significantly

increases astrocytic system x_C- activity within 24 hours (Fig. 5.7.). The exact mechanism of how neuronal conditioned media causes this functional increase of system x_C- in astrocytes requires further study.

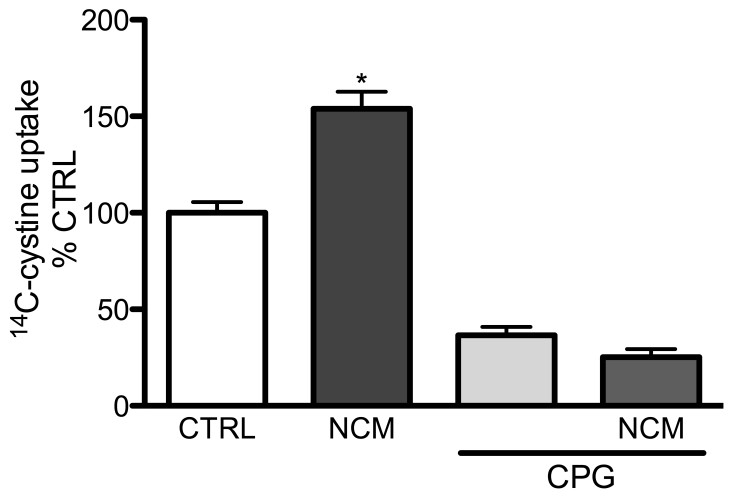

FIGURE 5.7. 24 hour exposure to neuronal-conditioned media (NCM) increases system x_C- mediated cystine uptake in astrocytes.

Culture media from the DIV 13 primary mouse glia-enriched cultures was replaced with NCM collected from DIV 13 neuronal-enriched cultures, ^{14}C-cystine uptake for 20 min is measured 24 hours later. Bars show % control (mean ± s.e.m., n = 12-16). * indicates significantly different from control uptake.

CONCLUSION

System x_C^- dysregulation has been implicated in various neurodegenerative diseases and psychiatric disorders. The dual function of system x_C^- awards it a distinct role in influencing cell fate. It may be protective by promoting cystine uptake and preventing oxidative stress, but it may be harmful by releasing glutamate and exacerbating excitotoxicity.

The goal of this thesis was to add to the knowledge regarding the role of system x_C^- in neuronal death. Two major advancements to the field were made. First, we discovered that the environmental neurotoxin BMAA acts as a substrate for system x_C^-. Through this action, BMAA both inhibits cystine uptake, leading to oxidative stress and drives glutamate release causing excitotoxicity. This result is important not only for understanding the mechanism of BMAA toxicity, but also provides evidence that compounds that are transported by system x_C^-, particularly if they do not lead to increased glutathione production, are potentially highly neurotoxic. Second, the results involving FGF-2 suggest that increasing system x_C^- selectively on astrocytes is potentially harmful to neurons. This finding, in concert with the well known neurotoxic action of glomas, that also have upregulated system x_C^-, suggests a general hypothesis that upregulation of system x_C^- on non-neuronal cells can be toxic to neurons.

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