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FGF-2 Induces Neuronal Death Through Upregulation of System xc-

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Abstract

The cystine/glutamate antiporter (system xc-) transports cystine into cell in exchange for glutamate. Fibroblast growth factor-2 (FGF-2) upregulates system xc- selectively on astrocytes, which leads to increased cystine uptake, the substrate for glutathione production, and increased glutamate release. While increased intracellular glutathione can limit oxidative stress, the increased glutamate release can potentially lead to excitotoxicity to neurons. To test this hypothesis, mixed neuronal and glial cortical cultures were treated with FGF-2. Treatment with FGF-2 for 48 h caused a significant neuronal deathin these cultures. Cell death was not observed in neuronal-enriched cultures, or astrocyte-enriched cultures, suggesting the toxicity was the result of neuron-glia interaction. Blocking system xc- eliminated the neuronal death as did the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), but not the NMDA receptor antagonist memantine. When cultures were exposed directly to glutamate, both NBQX and 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. The most likely explanation for the results is that 48 h FGF-2 treatment induces AMPA/kainate receptor toxicity through increased system xc- function resulting in increased release of glutamate. At the same time, FGF-2 alters the sensitivity of the neurons to glutamate toxicity in a manner that promotes selective AMPA/kainate receptor mediated toxicity.

Abbreviations

FGF-2 lactate dehydrogenase
LDH lactate dehydrogenase
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
CPG (s)-4-carboxyphenylglycine
SSZ sulfasalazine
NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NASPM 1-naphthyl acetyl spermine
GSH glutathione
EAAT excitatory amino acid transporter
TBOA DL-*threo*-β-Benzyloxyaspartic acid

Keywords

FGF-2, Excitotoxicity, AMPA, System xc-, Glutamate, Cystine

1. Introduction

Fibroblast growth factor 2 (FGF-2), despite its discovery in fibroblasts, is widely expressed throughout the brain (Eckenstein et al., 1991a, Dono, 2003). FGF-2 acts on members of the FGF receptor family leading to activation of multiple intracellular pathways, including the PI3K/Akt and MEK/ERK pathways (Reuss and Bohlen und Halbach, 2003) and 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. FGF-2 is upregulated by ischemic damage (Alzheimer and Werner, 2002) and traumatic brain injury (Mellergård et al., 2012), while FGF-2 treatment is protective in Alzheimer's disease models (Mark et al., 1997). The role of FGF-2 in psychological disorders is complex, with alteration in FGF-2, FGF-2 receptors, and FGF-2 signaling pathways in schizophrenia, addiction, and major depression (Flores and Stewart, 2000, Evans et al., 2004, Terwisscha van Scheltinga et al., 2010). FGF-2 was tested in clinical trials for the treatment for stroke but without success (Clark et al., 2000, Bogousslavsky et al., 2002). We have shown that FGF-2 upregulates system xc- (Liu et al., 2012), suggesting the possibility that some of the effects of FGF-2 may be through that action.

System xc- is a cystine/glutamate antiporter on the cell membrane (Sato et al., 1995). Physiologically, system xc- takes up cystine and releases glutamate 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 be used to synthesize 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 xc- can have multiple effects. Glutamate is the most important excitatory neurotransmitter in the central nervous system. Glutamate released from astrocytes is 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 overactivation

of NMDA receptors leading to excess calcium influx triggering cell death (Choi et al., 1987). An emerging theory is that activation of extrasynaptic NMDA receptors is particularly damaging (Hardingham and Bading, 2010).

System xc- is widely expressed in the central nervous system (Sato et al., 2002, Burdo et al., 2006). Cell culture studies have shown that immature neurons rely on system xc- 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 xc- 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 glutamate receptors leading to excitotoxicity (Choi et al., 1987). In mature cell cultures, neurons have low levels of system xc- activity, while astrocytes exhibit high levels of system xc- activity (Lobner, 2009). This shift of function indicates that as cells mature they take on more specialized tasks with astrocytes taking up cystine and releasing glutathione which is used by neurons (Fellin and Carmignoto, 2004, Stipursky et al., 2011, Suzuki et al., 2011). In contrast to the enhancement of neurotoxicity by releasing glutamate, system xc- activity on non-neuronal cells can be protective to neurons. Overexpression of xCT in astrocytes has been shown to enhance glutathione release and protect neurons from oxidative stress (Shih et al., 2006).

The dual actions of system xc- provide it with a unique potential for influencing cell fate. System xc- activity can be either beneficial or destructive depending on the cellular properties. If cells are undergoing oxidative stress, increasing system xc- activity should be protective as it increases cystine uptake, which contributes to increased antioxidant defense. However, if the cells are susceptible to excitotoxicity, increasing system xc- activity may be destructive by increasing extracellular glutamate and potentially causing excitotoxicity.

In the present study, we used primary mixed neuronal and glial cortical cell cultures to investigate the effect of 48 h FGF-2 treatment on neuronal survival/death. We have previously demonstrated that 24 h FGF-2 treatment upregulated system xc- activity on astrocytes. We demonstrate here that after system xc- is upregulated for a prolonged period of time, neuronal death occurs due to system xc- mediated excitotoxicity.

2. Results

2.1. Prolonged FGF-2 treatment induces neuronal death in mixed cortical cultures

As reported previously (Liu et al., 2012), 24 h FGF-2 treatment of mixed neuronal and glial cultures did not cause any significant cell death. However, significant neuronal death was observed after 48 h of FGF-2 treatment (Fig. 1).

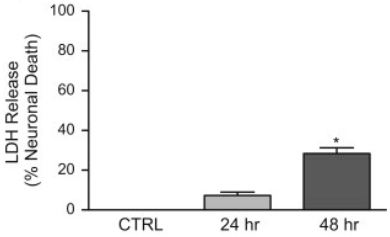

Fig. 1. FGF-2 induced toxicity occurs in mixed neuronal and glial cultures after 48 h treatment. Bars show % neuronal cell death (mean±s.e.m., *n*=24) quantified by measuring release of LDH, 24 and 48 h after the beginning of treatment with 100 ng/ml FGF-2. * indicates significant difference from control.

The cell death following FGF-2 treatment for 48 h was observed in mixed neuronal and glial cultures, but not neuronal-enriched cultures (Fig. 2A) or glial-enriched cultures (Fig. 2B), suggesting an interaction of glia and neurons is necessary for FGF-2 induced neuronal death to occur.

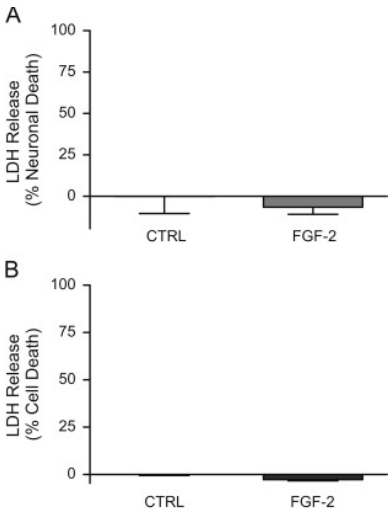

Fig. 2. FGF-2 does not induce toxicity after 48 h treatment in either neuronal-enriched or astrocyte-enriched cultures. A: Neuronal-enriched cultures. Bars show % neuronal death (mean±s.e.m., *n*==8) quantified by measuring release of LDH, 48 hours after the beginning of treatment with 100 ng/ml FGF-2. B: Astrocyte-enriched cultures. Bars show % cell death (mean±s.e.m., *n*=16) quantified by measuring release of LDH, 48 h after the beginning of treatment with 100 ng/ml FGF-2.

2.2. FGF-2 induced neuronal death is mediated by system xc-

We have shown previously that 24 h FGF-2 treatment significantly increased system xc- activity selectively in astrocytes, with no effect on neuronal-enriched and microglial-enriched cultures (Liu et al., 2012). To determine whether FGF-2 upregulated system xc- function was still present after 48 h we measured 20 min ^{14}C-cystine uptake following 48 h FGF-2 treatment of astrocyte-enriched cultures. FGF-2 treatment doubled the ^{14}C-cystine uptake, and the uptake was mediated by system xc- as its inhibitor(s)-4-carboxyphenylglycine (CPG) completely blocked the increase (Fig. 3).

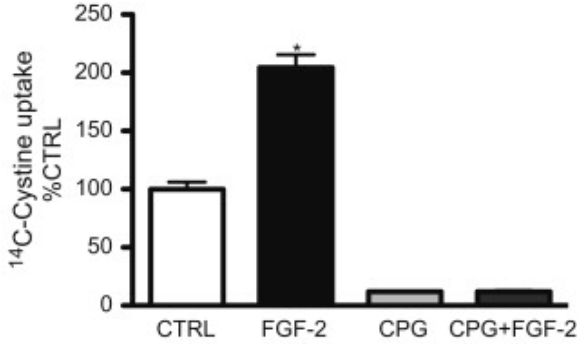

Fig. 3. FGF-2 induced increase in cystine uptake is mediated by system xc-. Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 48 h, washed into a growth factor free media, and ^{14}C-cystine uptake measured for 20 min with or without the system xc- antagonist (s)-4-carboxyphenylglycine (200 µM CPG). Bars show % control (mean±s.e.m., *n*=6). * indicates significant difference from control uptake.

To assess whether system xc- was involved in the FGF-2 induced neuronal death, we tested the effects of the system xc- inhibitors CPG and sulfasalazine (SSZ) on neuronal death induced by 48 h FGF-2 treatment. Cotreatment of cells with either CPG or SSZ during the 48 h FGF-2 incubation prevented the neuronal death (Fig. 4A). SSZ interferes with the LDH release assay, therefore the MTT metabolism assay was used to assess cell survival. While the effects of SSZ could not be tested by the LDH release assay, we did test CPG in this assay. Addition of CPG during the 48 h FGF-2 treatment was also protective by the LDH release assay (Fig. 4B).

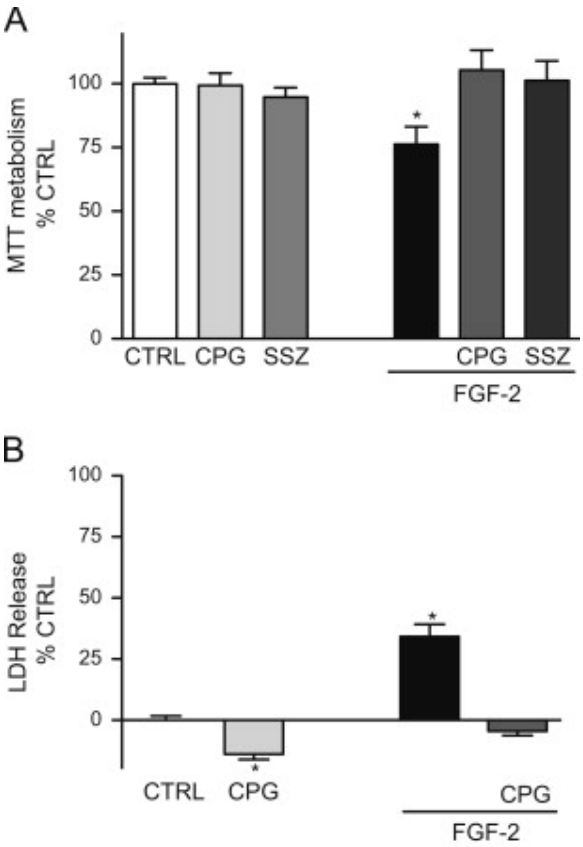

Fig. 4. FGF-2 induced neuronal death is prevented by blocking system xc-. Mixed neuronal and astrocytecultures were exposed to FGF-2 (100 ng/ml) with or without the system xc- antagonists (s)-4-carboxyphenylglycine (200 μM CPG) or sulfasalazine (300 μM SSZ) for 48 h. A: Bars show % cell survival(mean±s.e.m., *n*=8) quantified by measuring MTT reduction. B: Bars show % cell death (mean±s.e.m., *n*=8) quantified by measuring release of LDH. * indicates significant difference from control.

2.3. AMPA/kainate, but not NMDA receptors, mediated the neuronal death

System xc- mediates cystine uptake and glutamate release at the same time. Cystine uptake contributes to GSH synthesis which acts to decrease oxidative stress, therefore, it is unlikely that enhanced cystine uptake is responsible for the FGF-2 induced cell death. However, increasing glutamate release can lead to over-activation of glutamatergic receptors to cause excitotoxicity. To test this possibility, various glutamate receptor antagonists were tested to determine if they prevented FGF-2 induced neuronal death. The AMPA/kainate receptor antagonist NBQX, but not the NMDA receptor blocker memantine, blocked the FGF-2 induced neuronal death (Fig. 5A). In contrast to this result, both NBQX and memantine blocked neuronal death induced by direct addition of glutamate (Fig. 5B). 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. 6).

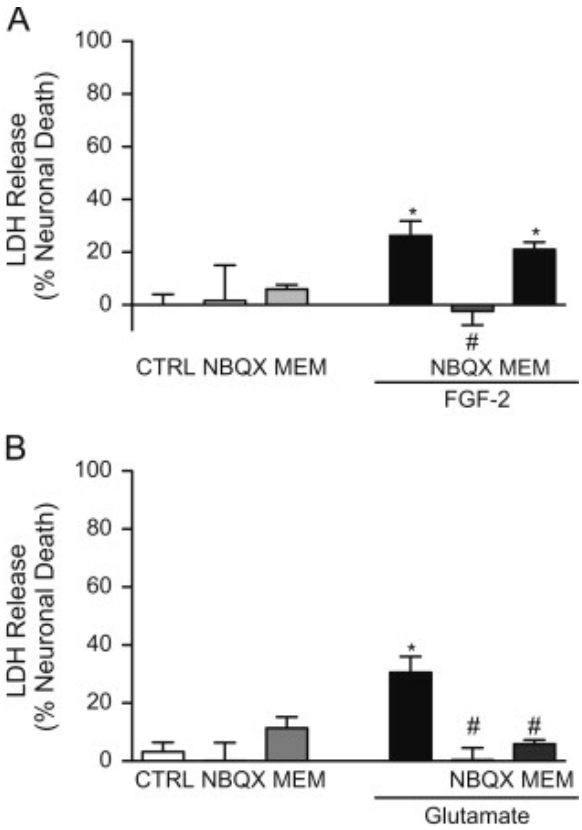

Fig. 5. Effects of the AMPA/kainate receptor antagonist NBQX or the NMDA receptor antagonist memantineon neuronal death induced by 48 hour exposure to FGF-2 or glutamate. A: FGF-2 (100 ng/ml) treatment induced toxicity is attenuated by blocking AMPA receptors. B: Glutamate (15 μM) induced toxicity is attenuated by blocking AMPA/kainate 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 h after the beginning of the insult. *indicates significant difference from control. # indicates significant difference from FGF-2 or glutamate treated.

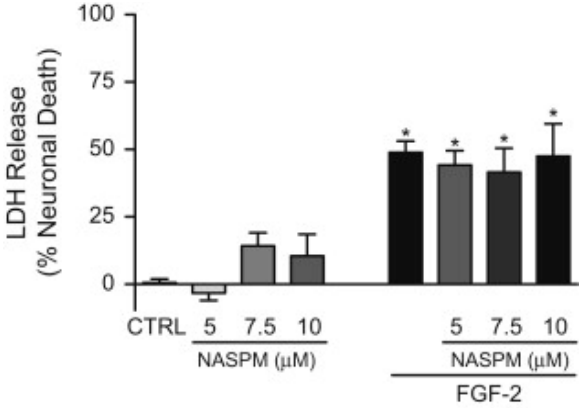

Fig. 6. FGF-2 induced toxicity is not mediated by calcium-permeable AMPA receptors. Mixed neuronal and glial cultures were exposed to FGF-2 (100 ng/ml) for 48 h with or without the calcium permeable AMPA receptor antagonist 1-naphthyl acetyl spermine (NASPM). Bars show % neuronal cell death (mean±s.e.m., *n*=8) quantified by measuring release of LDH, 48 hours after the beginning of the insult. * indicates significant difference from control.

2.4. FGF-2 does not alter EAAT function

One possibility for the glutamate receptor mediated neuronal death induced by FGF-2 is that it downregulates glutamate uptake leading to increased extracellular glutamate. We measured excitatory amino acid transporter (EAAT) activity using ^{3}H-d-aspartate. The uptake of ^{3}H-d-aspartate was attenuated by the general EAAT inhibitor DL-*threo*-β-Benzyloxyaspartic acid (TBOA). However, FGF-2 treatment for 24 or 48 h had no effect on ^{3}H-d-aspartate uptake (Fig. 7).

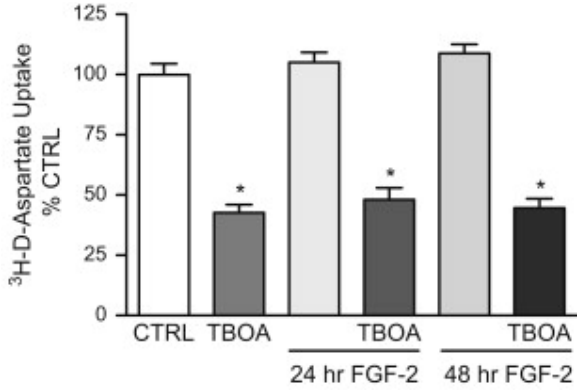

Fig. 7. FGF-2 does not alter EAAT function. Astrocyte-enriched cultures were exposed to FGF-2 (100 ng/ml) for 24 or 48 h, washed into a growth factor free media, and ^{3}H-d-aspartate uptake measured for 20 min with or without the general EAAT inhibitor dl-*threo*-β-Benzyloxyaspartic acid (100 μM TBOA) present. Bars show % control (mean±s.e.m., *n*=8). * indicates significant difference from control uptake.

3. Discussion

This study examined the mechanism by which FGF-2 induces neuronal cell death in mixed neuronal and glial cultures, focusing on the role of its selective upregulation of system xc- on astrocytes. System xc- is of great interest because of its dual transport properties. Through uptake it provides cystine to the cells, which is converted intracellularly to cysteine, which is the rate limiting component for the formation of glutathione, the major antioxidant in the brain (Dringen et al., 2000, Dringen and Hirrlinger, 2003). In contrast, system xc- mediated glutamate release can lead to uncontrolled extracellular glutamate levels and enhance excitotoxicity (Fogal et al., 2007, Jackman et al., 2010). Both oxidative stress and excitotoxicity are involved in various neurodegenerative diseases and psychological disorders. Also, complex patterns of 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 xc- function may be responsible for some of the actions of FGF-2.

A balance between glutamate release from system xc- and glutamate uptake by EAATs is likely to determine whether extracellular glutamate reaches toxic levels. This is particularly important since it has been shown previously that FGF-2 can increase EAAT expression (Figiel et al., 2003). However, we failed to observe an increase in EAAT activity as measured by ^{3}H-d-aspartate uptake. The lack of upregulation of EAAT function by FGF-2 in this system likely contributes to why FGF-2 induces toxicity.

Our previous study showed that 24 h FGF-2 treatment resulted in an increase in cystine uptake through system xc- on astrocytes, with no effect on neuronal and microglial cystine uptake (Liu et al., 2012). The FGF-2 treatment induced increase in system xc- was mediated through activation of FGFR1and required both MEK/ERK and PI3 kinase pathway activation. All of these cellular events happened without significant neuronal toxicity. However, in the current study we found that after 48 h FGF-2 treatment, significant neuronal cell death began to occur and this neuronal death was blocked by the system xc- antagonists CPG and SSZ.

The role of system xc- in cell death has been investigated in different cell types and under different conditions. Inhibition of system xc- has been shown to be harmful to system xc- expressing cells. Oxidative glutamate

toxicity was first described in immature neuronal cultures, in which system xc- is the major route of cystine uptake (Murphy et al., 1989, Murphy et al., 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 xc- levels would likely be protective.

However, increasing system xc- function is not always protective. Microglial system xc- activity has been shown to be toxic to surrounding cells (Domercq et al., 2007). Primary microglia activated by bacterial components have been shown to release enough glutamate through system xc- to kill neurons (Piani and Fontana, 1994) and oligodendrocytes (Domercq et al., 2005). The death of oligodendrocytes under these conditions was decreased by blocking AMPA/kainate receptors with CNQX (Domercq et al., 2005). This result is similar to what we have observed in primary mixed neuronal and glial cultures. The difference is that oligodendrocytes are normally sensitive to AMPA/kainate 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). Although, this is not always that case, as the AMPA/kainate receptor antagonist NBQX was neuroprotective in an in vivo model involving lipopolysaccharide plus cystine induced neuronal death (Kigerl et al., 2012).

Selectively increasing system xc- on astrocytes with IL-1*β* can also lead to increased glutamate release, which potentiates neuronal death induced by hypoglycemia and hypoxia (Jackman et al., 2010, Jackman et al., 2012), both of which are 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 and was also decreased by inhibiting system xc- (Fogal et al., 2007, Jackman et al., 2010, Jackman et al., 2012). Our data shows that FGF-2 treatment, like IL-1*β*, selectively increases system xc- activity on astrocytes (Liu et al., 2012). But FGF-2 treatment by itself induced neuronal cell death after system xc- activity was upregulated for 48 h. Also, the pharmacological profile of the excitotoxicity was different from non-FGF-2 treated neurons. While neuronal death induced by direct exposure of cultures to glutamate was blocked by either NMDA or AMPA/kainate receptor antagonists, FGF-2 induced neuronal death was only attenuated by the AMPA/kainate receptor antagonist. Interestingly, FGF-2 treatment has been shown to potentiate glutamate toxicity in PC-12 cells (Schubert et al., 1992).

FGF-2 has been shown 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. In cerebellar granule cells FGF-2 treatment caused a time-dependent decrease in expression of NMDA receptor subunitsGluN2A and GluN2C with a decrease in NMDA-evoked calcium influx, while GluN1 and GluN2B levels were not changed (Brandoli et al., 1998). FGF-2 pretreatment protected striatal neurons from NMDA receptor mediated toxicity (Freese et al., 1992), while systematic administration of FGF-2 protected against intrastriatal injection of NMDA (Nozaki et al., 1993a, Nozaki et al., 1993b). Also, 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 (Mattson et al., 1993). In contrast, FGF-2 treatment has been shown to increase AMPA receptor subunit GluA1 levels in the same cultures (Cheng et al., 1995). These results suggested the possibility that FGF-2 induced neuronal death may be due to increase in the levels of GluA2 subunit lacking AMPA receptors, which have high calcium permeability (Bannerman et al., 2007). However, the selective antagonist of these calcium permeable AMPA channels, NASPM, failed to offer protection in our study. At concentrations above 5 µM, NASPM began to induce some toxicity by itself. However, the IC_{50} of NASPM for calcium-permeable AMPA receptors is 0.33 µM (Brackley et al., 1993). Therefore, the 5 µM concentration that was not toxic should have been an effective concentration. The most likely explanation of the results is that increased glutamate release from system xc- causes an AMPA/kainate 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 for stroke, FGF-2 not only failed to show any beneficial effect, it actually increased the mortality rate (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 fully understood. One possibility is that the FGF-2 induced increase in system xc- activity, and the dual functions of system xc-, are responsible for the mixed actions of FGF-2 in the treatment of cerebral ischemia. The deciding factor for whether FGF-2 treatment is protective or injurious may be the importance of excitotoxicity and oxidative stress in each individual situation. FGF-2 administration has been shown to be beneficial in rodent studies in other disease models, such as depression (Turner et al., 2008c) and the rotenone model of Parkinson's disease (Hsuan et al., 2006). It is also possible that these beneficial effects may be mediated by the FGF-2 effect on system xc-, which leads to enhanced glutathione synthesis. In contrast, in the 6-hydroxydopamine model of Parkinson's disease there was decreased damage in mice lacking xCT (Massie et al., 2011). This result is consistent with system xc- function being damaging in that model.

The dual amino acid transport function of system xc- allow it to 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. While increasing cystine uptake can be protective by increasing the cells' antioxidant 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 xc- 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.

4. Experimental procedures

4.1. 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).

4.2. 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 h 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 Health 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.

4.3. Induction of neuronal death

All experiments were performed on cultures 13–14 days in vitro (DIV). Cultures were exposed to different compounds for 48 h in media as described for plating except without serum.

4.4. 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 h after the beginning of the insult. Control 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 h 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 (Koh and Choi, 1987, Lobner, 2000). Glial cell death (assessed by trypan blue staining) was not observed in any of the current studies involving mixed neuronal and glial cultures.

4.5. 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 h after the beginning of the insult to the cells. 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.

4.6. ^{14}C-cystine uptake

^{14}C-cystine uptake 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 (0.025 µCi/ml) for 20 min with or without CPG present. 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.

4.7. ^{3}H-d-aspartate uptake

To assess EAAT function, uptake of radiolabeled d-aspartate into cultures was measured. 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 ^{3}H-d-asparate (0.25 µCi/ml) for 20 min with or without 100 µM of the general EAAT inhibitor dl-*threo*-β-Benzyloxyaspartic acid (TBOA) present. Following ^{3}H-d-aspartate exposure, cultures were washed, dissolved, and scintillation counted as for ^{14}C-cystine studies.

4.8. 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 $p<.05$ being considered significant.
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