Non-cell autonomous influence of the astrocyte system $x_c^{-}$ on hypoglycaemic neuronal cell death

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ABSTRACT

Despite longstanding evidence that hypoglycaemic neuronal injury is mediated by glutamate excitotoxicity, the cellular and molecular mechanisms involved remain incompletely defined. Here, we demonstrate that the excitotoxic neuronal death that follows GD (glucose deprivation) is initiated by glutamate extruded from astrocytes via system $x_c^{-}$ – an amino acid transporter that imports L-cystine and exports L-glutamate. Specifically, we find that depriving mixed cortical cell cultures of glucose for up to 8 h injures neurons, but not astrocytes. Neuronal death is prevented by ionotropic glutamate receptor antagonism and is partially sensitive to tetanus toxin. Removal of amino acids during the deprivation period prevents – whereas addition of L-cystine restores – GD-induced neuronal death, implicating the cystine/glutamate antiporter, system $x_c$-. Indeed, drugs known to inhibit system $x_c^{-}$ ameliorate GD-induced neuronal death. Further, a dramatic reduction in neuronal death is observed in chimaeric cultures consisting of neurons derived from WT (wild-type) mice plated on top of astrocytes derived from svt mice, which harbour a naturally occurring null mutation in the gene (Slc7a11) that encodes the substrate-specific light chain of system $x_c^{-}$ (xCT). Finally, enhancement of astrocytic system $x_c^{-}$ expression and function via IL-1β (interleukin-1β) exposure potentiates hypoglycaemic neuronal death, the process of which is prevented by removal of L-cystine and/or addition of system $x_c^{-}$ inhibitors. Thus, under the conditions of GD, our studies demonstrate that astrocytes, via system $x_c^{-}$, have a direct, non-cell autonomous effect on cortical neuron survival.

Key words: aglycaemia, astrocyte, cystine, glutamate, neuronal death, non-cell autonomous.

INTRODUCTION

Hypoglycaemia is a medical emergency that arises as a serious complication of insulin therapy in diabetic patients. It is also prevalent in neonates, in patients with insulin-producing tumours, and can occur as a consequence of brain ischaemia. Severe hypoglycaemia, defined as less than 2 mM blood glucose, essentially renders the brain aglycaemic, leading to cognitive impairments and frank neuronal injury (Ryan et al., 1990; Langan et al., 1991; Suh et al., 2007a; Xu et al., 2011a). Evidence indicates that hypoglycaemic/aglycaemic neuronal cell death is mediated by glutamate excitotoxicity (Wieloch, 1985; Monyer et al., 1989). Following activation of glutamate receptors, a cascade of biochemical events is initiated that ultimately leads to neuronal cell death via processes dependent on reactive oxygen species, neuronal zinc release, activation of poly(ADP-ribose) polymerase-1, and alterations in mitochondrial function. Inhibition of these downstream targets of glutamate receptor activation show some success in reducing hypoglycaemic brain injury (for review, see Suh et al., 2007b). However, the cellular source and molecular mechanisms by which glutamate is released remain incompletely defined.

The contribution of both synaptic and non-synaptic sources of glutamate to excitotoxic neuronal injury under conditions of energy deprivation has been demonstrated. Yet, until recently, most therapeutic protective strategies have focused solely on the neuron, despite numerous studies that highlight the importance of the astrocyte to neuronal survival (Chen and Swanson, 2003). While its role in neuroprotection is best appreciated, several studies now demonstrate the contribution of the astrocyte to neuronal cell death (for review, see Barbeito et al., 2004; Lobsiger and Cleveland, 2007). With respect to this, recent work from our laboratory...
demonstrated that astrocyte system \( \text{x}^- \) (cystine/glutamate antiporter) activity contributes to hypoxic neuronal death via a glutamate-mediated mechanism (Fogal et al., 2007; Jackman et al., 2010b). System \( \text{x}^- \) is a heteromeric amino acid transporter consisting of two subunits: \( \text{xT}^- \) – the light chain conferring substrate specificity – and a heavy chain thought to target the transporter to the plasma membrane (Sato et al., 1999; Bassi et al., 2001). The import of cystine via system \( \text{x}^- \) is directly coupled to glutamate export, which occurs in a \( \text{Na}^+ \)-independent manner (Bannai and Kitamura, 1980). Enhanced system \( \text{x}^- \) activity has been previously reported to contribute to excitotoxic neuronal death in numerous other experimental paradigms as well (Piani and Fontana, 1994; Ye et al., 1999; Barger and Basile, 2001; Qin et al., 2006; Savaskan et al., 2008; Sontheimer, 2008; Massie et al., 2011). Although system \( \text{x}^- \) is functionally active in neurons, astrocytes and microglia (Burdo et al., 2006; Dun et al., 2006; Domercq et al., 2007; La Bella et al., 2007; Jackman et al., 2010b), herein, we describe astrocytic system \( \text{x}^- \) as the source of glutamate required for the initiation of non-cell autonomous neuronal death following GD (glucose deprivation) in vitro.

Part of the work has been published in abstract form (Jackman et al., 2010a).

MATERIALS AND METHODS

Cell culture

All media including media stock, glial plating medium, neuronal plating medium, mixed culture plating medium and maintenance medium have been fully described (Jackman et al., 2010b). The glucose-free BSS\(_p\) (balanced salt solution) contains: 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl\(_2\), 1 mM Na\(_2\)HPO\(_4\), 26.2 mM NaHCO\(_3\), 1.8 mM CaCl\(_2\), 0.01 mM glycine, 2 mM L-glutamine and 1 × MEM amino acids. For most experiments, BSS\(_p\) contains purchased 1 × MEM amino acids (Invitrogen); however, amino acids were reconstituted individually for the removal and addition experiments. With the exception of the concentration-response experiment, BSS\(_p\) contained 100 \( \mu \)M cystine, the standard concentration found in cell culture medium.

Primary astrocyte cultures were derived from cerebral cortices of day 1–3 postnatal CD1 mouse pups (Charles River) as described (Trackey et al., 2001). This plating procedure routinely produces monolayers of protoplasmic-like astrocytes, which following confluence are treated with 8 \( \mu \)M AraC once for 2 days to prevent glial cell growth. The medium was partially replenished (1/2 volume exchange) twice weekly. Experiments were performed on purified neuronal cultures after 7–10 days in vitro.

Primary neuronal cultures were derived from dissociated cortical cells of embryonic day 15 CD1 (Charles River) mouse fetuses (Brewer et al., 1993). Two days after plating in neuronal plating medium, cultures were treated with 1 \( \mu \)M AraC once for 2 days to prevent glial cell growth. The medium was partially replenished (1/2 volume exchange) twice weekly. Experiments were performed on purified neuronal cultures after 7–10 days in vitro.

Mixed cortical cell cultures containing an approximate 50:50 neuron to astrocyte ratio were prepared by culturing dissociated cells from embryonic day 15 mouse fetuses on to a confluent layer of microglia-depleted astrocytes in mixed culture plating media as described in detail (Trackey et al., 2001; Jackman et al., 2010b). Experiments were performed on mixed cortical cultures after 13–14 days in vitro.

GD

Mixed cortical cell cultures were washed thoroughly (8 × 750 \( \mu \)l) into BSS\(_p\). Glucose (final concentration=10 mM) was immediately added to parallel cultures to serve as controls and at times added back to experimental conditions as indicated in each Figure legend. It should be noted that during the course of the experiments, we found that death measured at 8 h post-aglycemia was essentially the same as that measure 20–24 h later following a glucose ‘rescue’, indicating that cell death was complete at 8 h and the glucose add-back was unnecessary and non-helpful. Inhibitors – with the exception of an overnight incubation with tetanus toxin – were given at the initiation of GD.

Measurement of cell death

Cell death was quantitatively determined by spectrophotometric measurement of LDH (lactate dehydrogenase) as described previously (Uliasz and Hewett, 2000). Neuronal cell death is expressed as a percentage of total neuronal LDH activity (defined as 100%) determined by exposing parallel cultures to 250 \( \mu \)M NMDA (\( N \)-methyl-\( \alpha \)-aspartate) (20–24 h). Since cultured cortical astrocytes neither express NMDA receptors (Backus et al., 1989; Chan et al., 1990; Janssens and Lesage, 2001; B. Fogal and S.J. Hewett, unpublished data) nor are injured by GD up to 8 h (Figure 1, inset), changes in LDH
activity can reasonably be used as a specific marker of neuronal death. In most but not all cases, 'basal' LDH released from control cultures (attrition due to extensive washes) was subtracted from values obtained in experimental conditions to yield the signal specific to hypoglycaemic death. To quantify astrocyte death, astrocyte release of LDH under control and experimental conditions was expressed as a percentage of total astrocyte LDH activity (defined as 100%), determined by exposing parallel cultures to 5 μM Calphostin C for 20–24 h (Ikemoto et al., 1995).

IL-1β (interleukin-1β) treatment
To selectively enhance astrocytic system xCT expression/activity (Jackman et al., 2010b), cultures were treated with 0.01–1 ng/ml recombinant murine IL-1β (R&D Systems) in an incubation buffer of media stock supplemented with 0.1% fatty-acid-free BSA (Sigma). The vehicle used in all other conditions was media stock supplemented with 0.1% fatty-acid-free BSA.

qPCR (quantitative PCR)
qPCR was performed using mouse-specific primer pairs [Taqman Gene Expression Assays, Applied Biosystems: xCT (Mm00442530_m1)] per manufacturer’s instructions using the comparative cycle threshold method (ΔΔCT) with β-actin as the housekeeping control as described (Jackman et al., 2010b). Importantly, β-actin Ct values were unaffected by IL-1β treatment. Amplification efficiency was >94%.

RESULTS
As reported previously, selective neuronal degeneration occurred in a time-dependent manner in mixed cortical cell culture following GD (Figure 1), whereas purified astrocytes (Figure 1, inset) and astrocytes in mixed cultures (Figures 4b and 4d) were resistant for up to 8 h, the longest time-point assessed (Monyer and Choi, 1988; Monyer et al., 1989; Goldberg and Choi, 1993). Also in agreement with previous studies (Monyer and Choi, 1988; Monyer et al., 1989), neuronal injury was prevented by ionotropic glutamate receptor antagonism (Figure 2). The small amount of death that is found in control cultures (1–10%) reflects LDH release that results from the extensive washing (i.e. wash damage) that is used to render the cultures aglycaemic.

Figure 1 GD injures neurons, but not astrocytes in vitro
Mixed cortical cultures or purified astrocytes (inset) were washed into an incubated medium containing (hatched bars) or lacking (black bars) glucose. The percentage of total cell death was determined at the times indicated. Between group differences (*) were determined by one-way (astrocytes) or two-way ANOVA (mixed cultures) followed by Bonferroni’s post-hoc test (n=11–12 cultures from four independent experiments).

Figure 2 Hypoglycaemic neuronal cell death is attenuated by glutamate receptor antagonism
Mixed cortical cultures were washed into a BSS0 containing vehicle, MK-801 (10 μM) or MK-801 plus CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 30 μM) for 8 h (GD). Neuronal cell death was determined 20–24 h later. (*) Indicates values significantly different from control conditions (10.56±3.02%) determined 24 h following wash of cells into BSS0 followed by immediate addition of glucose. (#) Represents a significant diminution of GD-induced cell death as determined by one-way ANOVA followed by Student–Newman–Keul’s post-hoc test (n=11 cultures from three experiments).
In support of this contention, we find that hypoglycaemic neuronal cell death occurred only in buffer containing amino acids, with an absolute requirement for l-cystine (Figure 5). Indeed, neuronal cell death was completely prevented when l-cystine alone was lacking during the deprivation period (Figure 5A), whereas addition of only l-cystine allowed hypoglycaemic neuronal cell death to ensue (Figure 5B). Although the concentration of l-cystine routinely found in tissue culture medium (100 μM) resulted in maximal cell death, significant injury was observed at 25 μM (Figure 5C), which is nearly identical with the calculated $K_m$ for $^{14}$C-l-cystine uptake into our cultures (Fogal et al., 2007). Removal and/or addition of l-methionine had no effect (Figures 5A and 5B), attesting to the specificity of the response for l-cystine. Methionine was chosen because, like cystine, it can be converted in the brain into cysteine via transulfuration (Vitvitsky et al., 2006).

In our cultures, astrocytes and neurons express xCT and functional system $x^-_c$ activity (Jackman et al., 2010b) in an apparent 2:1 ratio (Figure 6). Thus, to determine whether cell-autonomous or non-cell autonomous alterations in system $x^-_c$ activity underlie the initiation and/or progression of neuronal cell death, we prepared chimaeric cultures consisting of WT neurons plated on top of astrocytes derived from mice harbouring a null mutation in Slc7a11 (sut gene), which encodes xCT, the light subunit of system $x^-_c$ (Chintala et al., 2005), and then deprived them of glucose. In comparison with cultures containing both WT neurons and astrocytes, neuronal cell death following GD was substantially reduced in chimaeric cultures (Figure 7). Notably, WT neurons plated on sut astrocytes were equally sensitive to injury invoked by NMDA exposure (Figure 7, inset; 100% neuronal death). Additionally, the comparable LDH values measured following NMDA exposure demonstrate that neurons plated on WT or sut astrocytes had similar growth properties/cell densities. Hence, the differences in hypoglycaemic cell death observed when neurons were plated on sut astrocytes may not be explained by alterations in cell density nor by a global reduction in neuronal susceptibility to an excitotoxic insult. Finally, selective enhancement of xCT mRNA expression (Figure 8A) and xCT protein expression in astrocytes and not neurons (Jackman et al., 2010b) following IL-1β treatment resulted in a potentiation of hypoglycaemic neuronal cell death (Figure 8B). This IL-1β-potentiated hypoglycaemic neuronal cell death was blocked by the use of the system $x^-_c$ antagonists, 4-CPG and LY367385, and/or by removal of the system $x^-_c$ substrate,
Astrocytes initiate aglycaemic injury

Figure 5 Hypoglycaemic neuronal death is dependent on l-cystine
(A) Mixed cultures were deprived of glucose (4 h) in a medium containing (GD) or lacking (–AA) MEM amino acids, l-cystine (–CYSS) alone or l-methionine (–MET) alone. (*) Indicates values significantly different from control (mean ± 0.55 ± 0.28%); (≠) represents a significant diminution from GD-induced neuronal death as determined by one-way ANOVA followed by Student–Newman–Keul’s post-hoc test (n=24 cultures from six independent experiments). (B) Cultures were deprived of glucose (4 h) in a medium containing (GD) or lacking (–AA) MEM amino acids save for supplementation with 100 μM l-cystine (+CYSS) or 100 μM l-methionine (+MET). (*) Indicates values different from control (mean ± 2.87 ± 0.38% death), while (≠) represents a significant diminution from GD-induced neuronal death as determined by one-way ANOVA followed by Student–Newman–Keul’s post-hoc test (n=4). (C) Cultures were deprived of glucose for 8 h in a medium containing MEM amino acids and various concentrations of cystine (0–100 μM), after which neuronal death was assessed. (*) Indicates values significantly different from control conditions (mean ± 7.11 ± 1.27%) as determined by one-way ANOVA followed by Dunnett’s post-hoc test (n=4).

Figure 6 System xCT expression and activity is higher in astrocytes than in neurons
(A) Total RNA was isolated from unstimulated pure astrocytes and pure neurons (n=3 cultures each from three independent experiments), reverse transcribed and relative basal expression of xCT mRNA was assessed via qPCR. Data are expressed as means ± S.E.M. fold change in mRNA compared with pure astrocyte cultures (set at 1). No statistical difference was noted. (B) Pure astrocytes (n=12 cultures from three independent experiments) and pure neurons (n=8 cultures from two independent experiments) were washed and incubated with an uptake buffer containing [14C]-l-cystine (3 μM) for 30 min. Data are expressed as means ± S.E.M. [14C]-l-cystine uptake in pmol/30 min/mg protein. An asterisk (*) denotes values different from neurons as assessed by a Student’s t test. Significance was set at P<0.05.

l-cystine (Figure 8C). All together, these data are consistent with the obligate requirement of astrocytic system xCT in hypoglycaemia-induced excitotoxic neuronal cell death in this paradigm.

DISCUSSION

When blood glucose concentrations fall below 2 mM (normal=4–7 mM), brain glucose levels approach zero (Choi et al., 2001), precipitating neuronal injury (Ryan et al., 1990; Langan et al., 1991; Suh et al., 2007b; Xu et al., 2011b). Neurons, especially those residing in the hippocampus and cortex, are highly sensitive to GD (Auer et al., 1984; Monyer and Choi, 1988; Goldberg and Choi, 1993), whereas astrocytes have been demonstrated to be more resistant (Monyer and Choi, 1988; Monyer et al., 1989; Goldberg and Choi, 1993; Lyons and Kettenmann, 1998) (Figure 1). This is in accordance with the findings that rapid
ATP depletion occurs exclusively in neurons following GD in vitro (Choi et al., 2008) and that astrocytes contain glycogen stores (Cataldo and Broadwell, 1986; Swanson et al., 1990) that can be metabolized to meet their own metabolic needs (Swanson et al., 1990; Erecinska and Silver, 1994; Dienel and Cruz, 2006; Walls et al., 2009). Additionally, the ability to convert glutamate to pyruvate provides another possible mechanism whereby the tricarboxylic acid cycle in astrocytes can be maintained when levels of glucose are low (Bakken et al., 1985). Despite this, neuronal cell death does not appear to be a direct result of energy failure. In fact, several studies demonstrate that hypoglycaemic neuronal injury occurs secondary to glutamate excitotoxicity, as insulin-induced hypoglycaemia results in glutamate accumulation in the rat hippocampus and striatum (Sandberg et al., 1986; Silverstein et al., 1990) and in the cerebrum of the pig (Ichord et al., 1999) as measured by microdialysis. Moreover, ionotropic glutamate receptor antagonists protect against injury both in vivo and in vitro (Wieloch, 1985; Monyer and Choi, 1988; Monyer et al., 1989; Nellgard and Wieloch, 1992; Tasker et al., 1992; Ichord et al., 2001) (Figure 2). Nevertheless, questions concerning the cellular source and molecular mechanisms surrounding glutamate release following hypoglycaemia remain.

In vivo deafferentation studies suggest a neuronal contribution to hypoglycaemic neuronal cell death (Wieloch et al., 1985). Consistent with the work of Choi and co-workers (Monyer et al., 1992), we found partial but significant protection with tetanus toxin (3 μg/ml), suggesting that exocytotic release of glutamate may contribute slightly to hypoglycaemic neuronal cell death. However, it should be noted that 300 ng/ml – a concentration that we have previously shown to cleave neuronal synaptobrevin and block depolarization-induced glutamate release as well as high K⁺-induced neuronal cell death (Taylor and Hewett, 2002; Fogal et al., 2005), slightly but non-significantly attenuated neuronal death in our hands (Figure 3). Certainly, the effect of tetanus toxin at this concentration was not as robust as that observed following pharmacological inhibition of system xᵦ⁻ c (Figure 4) or via removal of its substrate, l-cystine (Figure 5). Although selective inhibitors for system xᵦ⁻ c are not available at this time, two CPG derivatives, best known for their ability to inhibit mGluR1 (i.e. 4-CPG and LY367385), were used to inhibit this transporter (Gochenauer and Robinson, 2001). A role for mGluR1 is unlikely, because the selective mGluR1 antagonist, YM298198, did not protect in this injury model. A role for mGluR5 is also unlikely as LY367385 (unlike 4-CPG) has no effect on mGluR5 (Watkins and Collingridge, 1994; Brabet et al., 1995; Kingston et al., 2002; Patel et al., 2004). These pharmacological studies and data obtained from sut-derived chimaeric cultures (Figure 7) implicate astrocytic system xᵦ⁻ c, which translocates glutamate into the extracellular space. While elimination of astrocytic system xᵦ⁻ c is sufficient to prevent GD-induced neuronal death, we also found that enhancement of astrocytic system xᵦ⁻ c could potentiate injury (Figure 8). Although IL-1β was utilized as a tool in this study, and is capable of altering the expression of other cellular proteins besides astrocyte xCT, our data demonstrating that its injury potentiating effects are dependent on cystine and blocked by system xᵦ⁻ c pharmacological antagonism (Figure 8) argue for the primacy of system xᵦ⁻ c over other potential targets. Our previous data, showing no effect of IL-1β on neuronal xCT expression or function, points to the central role of the astrocyte (Jackman et al., 2010b). Additionally, it is notable that diabetics have increased IL-1β serum levels compared with healthy individuals (Dogan et al., 2006). Given the increasing evidence of cross-talk between the peripheral immune and CNSs (central nervous systems), it is intriguing to speculate that there may be some physiological relevance (Figure 8).

An important caveat with respect to this study is the lack of information regarding the extracellular concentrations of cystine that exist either basally or under pathological conditions in the cerebral cortex. Herein, we demonstrate neuronal cell death with cystine concentrations at or above 25 μM (Figure 5C). This is in good agreement with the effective Km of system xᵦ⁻ c, which has been estimated in numerous in vitro preparations – including our own (≈30 μM) – to be in the range of 10–100 μM (Bannai and Kitamura, 1982; Hosoya et al., 2002; McBean, 2002; Fogal et al., 2007; Bridges, 2012). However, microdialysis measurements made in the nucleus accumbens demonstrate that the extracellular l-cystine concentration may be rather low (0.1–0.2 μM) (Baker et al., 2003), which some investigators interpret to mean that system
Figure 8 Enhanced astrocyte system x\textsubscript{c}\textsuperscript{-} activity potentiates hypoglycaemic neuronal death

(A) Purified astrocytes (n=3 from three independent experiments) were incubated with IL-1\textbeta or vehicle (media stock supplemented with 0.1% fatty-acid-free BSA) for 6 h, after which xCT mRNA expression was assessed. (*) Denotes values different from 0 h as determined by one-way ANOVA followed by Dunnett’s post-hoc test. [B] Mixed cultures were incubated with IL-1\textbeta for 20–24 h then washed into BSS, glucose was added after 3.5 h and neuronal cell death determined 20–24 h later. (*) Indicates values different from control (0 ng/ml IL-1\textbeta) as determined by one-way ANOVA followed by Dunnett’s post-hoc test (n=16 cultures from four independent experiments). (C) Mixed cultures were incubated with IL-1\textbeta (GD=IL-1\textbeta) or vehicle (GD) for 20–24 h, then washed into BSS, containing 4-CPG (50 \mu M) or LY367385 (50 \mu M) or one lacking cystine (−CYSS) for 4 h. (*) Indicates values different from GD. (#) Represents a significant diminution from the IL-1\textbeta-mediated potentiating effect of GD-induced neuronal death as determined by one-way ANOVA followed by Student–Newman–Keuls’s post-hoc test (n=6–16 cultures from four independent experiments).
response to an oxidative challenge, it also holds the potential to exacerbate CNS pathology via extrusion of glutamate into the extracellular space. As such, it is becoming increasingly clear that the consequences of system \( x_c^- \) activity are context-dependent whereby import of cystine is physiologically beneficial (Tanaka et al., 1999; Shih et al., 2003; Jakel et al., 2007) and/or can contribute to pathophysiology under certain conditions (Piani and Fontana, 1994; Ye et al., 1999; Barger and Basile, 2001; Qin et al., 2006; Fogal et al., 2007; Savaskan et al., 2008; Sontheimer, 2008; Jackman et al., 2010b; Massie et al., 2011).

The chimaeric cultures were integral to our position that glutamate efflux from astrocytes via system \( x_c^- \) contributed to GD-induced injury. Yet the precise mechanism by which system \( x_c^- \) activity links to glutamate-mediated neuronal injury is yet to be determined. It is possible that cellular changes in system \( x_c^- \) expression or function occur under hypoglycaemic conditions. With respect to the former, we did find that xCT mRNA expression in mixed cultures was increased in a time-dependent manner following GD. However, a statistically significant increase in xCT mRNA (~4-fold) did not occur until 8 h after GD (Supplementary Figure S1 available at http://www.asnneuro.org/an/004/an004e074add.htm). Given that much, if not all, of the death has already occurred by this time point, the relevance of this to the injury mechanism is questioned. Further, it is important to point out that it may not be necessary for enhanced release of glutamate to occur because glutamate concentrations needed to kill energy-deprived neurons are far less than those required to kill healthy neurons (Novelli et al., 1988). Additionally, it is possible that under conditions of hypoglycaemia, glutamate uptake is impaired, allowing system \( x_c^- \) derived glutamate to accumulate in the extracellular space. However, there is a body of literature demonstrating no change in glutamate uptake in primary mouse cortical astrocytes following 2 h of GD (Bakken et al., 1998) and only a 20% loss after 24 h (Swanson and Benington, 1996). Finally, it is possible that following the loss of system \( x_c^- \) function in the chimaeric cultures, its substrate, L-cystine, is now fully available for neuronal transport, perhaps yielding increased levels of neuronal GSH that could enhance neuronal survival subsequent to aglycaemic insult. However, this mechanism cannot explain the protective effect demonstrated following pharmacological inhibition of system \( x_c^- \) as both neurons and astrocyte transport are inhibited. Additionally, studies show that neurons rely more heavily on cysteine rather than cystine uptake for GSH biosynthetic needs (Kranich et al., 1996) and that neuronal GSH content is intimately related to astrocytic GSH levels secondary to extrusion of GSH from astrocytes (Hirrlinger et al., 2002).

Regardless of the precise mechanism, our data demonstrate that inhibition of system \( x_c^- \) through pharmacological means is sufficient to dramatically reduce neuronal cell death occurring secondary to GD and that genetic loss of astrocyte xCT recapitulates these findings. Conversely, the enhancement of astrocyte system \( x_c^- \) activity that occurs following IL-1β treatment exacerbates injury and facilitates neuronal cell death. More broadly, these data highlight the critical role of the astrocyte in non-cell autonomous excitotoxic hypoglycaemic neuronal cell death and further underscore their potential to serve as therapeutic targets for reducing excitotoxic neuronal injury in vivo. Importantly, this study adds to the burgeoning literature detailing the contribution of astrocytes to acute neuronal injury (Fogal et al., 2007; Jackman et al., 2010b) as well as in a variety of neuropathological states (for review, see Barbeito et al., 2004; Lobsiger and Cleveland, 2007). Acknowledging the limitations of cell culture models, we suggest that more detailed studies designed to assess the role of system \( x_c^- \) in hypoglycaemic neurodegeneration in vivo are warranted.

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**REFERENCES**

Auer RN, Wienoch T, Olsson Y, Siesjo BK (1984) The distribution of hypoglycemic brain damage. Acta Neuropath 64:177–191.

Augustin H, Grosjean Y, Chen K, Featherstone DE (2007) Nonvesicular release of glutamate by glial xCT transporters suppresses glutamate receptor clustering in vivo. J Neurosci 27:111–123.

Backus KH, Kettenmann H, Schachner M (1989) Pharmacological characterization of the glutamate receptor in cultured astrocytes. J Neurosci Res 2:274–282.

Baker DA, Madayag A, Kristiansen LV, Meador-Woodruff JH, Haroutunian V, Raju I (2008) Contribution of cystine-glutamate antiporters to the psychotomimetic effects of phencyclidine. Neuropsychopharmacology 33:1760–1772.

Baker DA, McFarland K, Lake RW, Shen H, Tang XC, Toda S, Kalivas PW (2003) Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nat Neurosci 6:743–749.

Baker DA, Shen H, Kalivas PW (2002a) Cystine/glutamate exchange serves as the source for extracellular glutamate: modifications by repeated cocaine administration. Amino Acids 23:161–162.

Baker DA, Xi ZX, Shen H, Swanson CJ, Kalivas PW (2002b) The origin and neuronal function of in vivo nonsynaptic glutamate. J Neurosci 22:9134–9141.

Bakken JJ, White LR, Unsgaard G, Aasly J, Sonnewald U (1998) [U-13C]glutamate metabolism in astrocytes during hypoglycemia and hypoxia. J Neurosci Res 51:636–645.

Bannai S (1984) Transport of cystine and cysteine in mammalian cells. Biochim Biophys Acta 779:289–306.

Bannai S, Kitamura E (1980) Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. Biochim Biophys Acta 625:110–110.

Bannai S, Kitamura E (1981) Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. Biochim Biophys Acta 671:1–10.

Bannai S, Kitamura E (1982) Adaptive enhancement of cystine and glutamate uptake in human diploid fibroblasts in culture. Biochim Biophys Acta 671:1–10.

Bannai S, Sato H, Ishii T, Sugita Y (1989) Induction of cystine transport activity in human fibroblasts by oxygen. J Biol Chem 264:18480–18484.

Barbeito LH, Pehar M, Cassina P, Vargas MR, Peluffo H, Viera L, Estevez AG, Beckman JS (2004) A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. Brain Res Brain Res Rev 47:263–274.
Banger SW, Basile AS (2001) Activation of microglia by secreted amyloid precursor protein evokes release of glutamate by cysteine exchange and attenuates synaptic function. J Neurochem 76:846–854.

Bassi MT, Gasol E, Manzoni M, Pineda M, Riboni M, Martin R, Zorzano A, Borsani G, Palacin M (2001) Identification and characterisation of human xCT that co-expresses, with 4F2 heavy chain, the amino acid transport activity system x−c−. Pflugers Arch 442:286–296.

Brabet I, Mary S, Bockaert J, Pin JP (1995) Phenylglycine derivatives discriminate between mGluR1- and mGluR5-mediated responses. Neuropharmacology 34:895–903.

Breger GJ, Torricelli JR, Eegei AK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576.

Bridges C, Keoka R, Wang H, Prasad P, Mehta P, Huang W, Smith S, Ganapathy V (2001) Structure, function, and regulation of human cysteine/glutamate transporter in retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 42:47–54.

Bridges RJ (2012) System x−c− cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. Br J Pharmacol 165:20–34.

Brigham MP, Stein WH, Moore S (1960) The Concentrations of cysteine and cysteine in human blood plasma. J Clin Invest 39:1633–1638.

Burdo J, Dangsch R, Schubert D (2006) Distribution of the cysteinyl-glutamate antiporter system x−c− in the brain, kidney, and duodenum. J Histochim Cytochem 54:549–557.

Cataldo AM, Broadwell RD (1986) Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. J. Neurons and glias. J Electron Microsc Technol 3:413–437.

Cavaler P, Hammar M, Rossi D, Mabou P, Attanasio O (2005) Tonic excitation and inhibition of neurons: ambient transmitter sources and computational consequences. Prog Biophys Mol Biol 87:3–16.

Chan PH, Chu L, Chen S (1990) Effects of MK-801 on glutamate-induced swelling of astrocytes in primary cell culture. J Neurosci Res 25:87–93.

Chen Y, Swanson RA (2003) Astrocytes and brain injury. J Cereb Blood Flow Metab 23:137–149.

Chintala S, Li W, Lamoreux ML, Ito S, Wakamatsu K, Sviderskaya EV, Bennett NW (1999) Reactive oxygen species mediate glutamate toxicity to oligodendrocytes. J Neurosci Res 33:1290–1300.

Choi IY, Lee SP, Kim SG, Gruetter R (2001) The influence of excitatory amino acid antagonists on calcium-depleted cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci 13:3510–3524.

Choi JW, Shin CY, Choi MS, Yoon SY, Ryu JH, Lee JC, Kim WK, El Kouni MH, Ko KY (2008) UTP protects cortical neurons from glucose deprivation-induced death: possible role of uridine phosphorylase. J Neurotrauma 25:695–707.

Clapp-Lilly KL, Roberts RC, Duffy LK, Irons KP, Hu Y, Drew KL (1999) An ultrastructural analysis of tissue surrounding a microdialysis probe. J Neurosci Methods 90:129–142.

Crawhall JC, Lietman PS, Schneider JA, Sezgmiiller JE (1968) Cystinosis. Plasma cysteine and cysteine concentrations and the effect of D-penicillamine and dietary treatment. Am J Med 44:330–339.

Da Silva WA, Fuchikawa M, Tinoco J, Lamas P, Deificio F (2000) Microglial role in excitotoxicity and neurodegeneration. Microsc Res Tech 51:151–252.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105.

De Bundel D, Schaller D, Ogier F, Yamauchi K, Terasaki T (2002) Enhancement of L-cystine transport activity and its relation to xCT gene induction at the blood-brain barrier by diethyl maleate treatment. J Pharmacol Exp Ther 302:225–231.

Ichord RN, Johnston MV, Traystman RJ (2001) MK801 decreases glutamate release and oxidative metabolism during hypoglycemic coma in piglets. Brain Res Dev Brain Res 128:139–148.

Ichord RN, Northington FJ, van Wyden D, Johnston MV, Kwon C, Traystman RJ (1999) Brain O2 consumption and glutamate release during hypoglycemic coma in piglets is temperature sensitive. Am J Physiol Heart Circ Physiol 276:H2053–H2062.

Ikemoto H, Tani E, Matsumoto T, Nakano A, Furumaya J (1995) Apoptosis of human glioma cells in response to calphostin C, a specific protein kinase C inhibitor. J Neurosurg 83:1008–1016.

Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gahwiler BH, Gerber U (1999) Inhibition of uptake ummsk rapid extracellular turnover of glutamate of nonvesicular origin. Proc Natl Acad Sci USA 96:8733–8738.

Jackman NA, Melchior SE, Hewett JA, Hewett SJ (2010a) An obligate role for astrocytic transport system x−c− in hypoglycemia-induced neuronal cell death. Program No. 156.3. 2010 Neuroscience Meeting Planner. Society for Neuroscience, San Diego, CA.

Jackman NA, Uliasz TF, Hewett SJ, Hewett JA (2010b) Regulation of system x−c− activity and expression in astrocytes by interleukin-1beta: implications for hypoxic neuronal injury. Glia 58:1806–1815.

Jakel RJ, Townsend JA, Kraft AD, Johnson JA (2007) Nrf2-mediated protection against 6-hydroxydopamine. Brain Res 1144:192–201.

Janssens N, Lesage AS (2001) Glutamate receptor subunit expression in primary neuronal and secondary glial cultures. J Neurochem 77:1457–1474.

Jones DP, Mody VC Jr, Carlson JL, Lynn MJ, Sternberg P Jr, (2002) Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. Free Radical Biol Med 33:1290–1300.

Kau KS, Madayag A, Mantsch JR, Gier MD, Abdulhameed O, Baker DA (2008) Blunted cystine-glutamate antiporter function in the nucleus accumbens promotes cocaine-induced drug seeking. Neuroscience 155:261–272.

Kingston AE, Griffey K, Johnson MP, Chamberlain MJ, Kelly G, Tomlinson R, Mody VC Jr, Carlson JL, Lynn MJ, Sternberg P Jr., (2002) Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. Free Radical Biol Med 33:1290–1300.

Krantz OG, Hamplecht B, Dringen R (1996) Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and axoglial cells derived from rat brain. Neurosci Lett 219:211–212.

La Bella V, Valentinis F, Piccoli T, Piccoli F (2007) Expression and developmental regulation of the cysteine/glutamate exchanger (x−c−) in the rat. Neurochem Res 32:1081–1090.
Langan SJ, Deary UJ, Hegbum DA, Frier BM (1991) Cumulative cognitive impairment following recurrent severe hypoglycaemia in adult patients with insulin-treated diabetes mellitus. Diabetologia 34:337–344.

Leweren J, Albrecht P, Tien ML, Henke N, Karunambaryam S, Kornblum HI, Wiedau-Pazos M, Schubert D, Maher P, Metnzer A (2009) Induction of Nrf2 and xCT are involved in the action of the neuroprotective antibiotic cephalaxin in vitro. J Neurochem 111:332–343.

Lobigsberg CS, Cleveland DW (2007) Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. Nat Neurosci 10:1355–1360.

Lyon SA, Kettenmann H (1998) Oligodendrocytes and microglia are selectively vulnerable to combined hypoxia and hypoglycaemia in vitro. J Cereb Blood Flow Metab 18:521–530.

Massie A, Schallier A, Kim SW, Fernando R, Kobayashi S, Beck H, De Bundel D, Vermeoseng K, Bannai S, Smolders I et al. (2011) Dopaminergic neurons of system x(N) – deficient mice are highly protected against 6-hydroxydopamine-induced toxicity. FASEB J 25:1359–1369.

Matyash V, Kettenmann H, Staelens J (2010) Heterogeneity in astrocyte morphology and physiology. Brain Res Rev 63:2–10.

McBean GJ (2002) Cerebral cystine uptake: a tale of two transporters. Trends Pharmacol Sci 23:299–302.

Melendez RI, Vuthiganon J, Kalivas PW (2005) Regulation of extracellular glutamate in the prefrontal cortex: focus on the cystine glutamate exchanger and I metabotropic glutamate receptors. J Pharmacol Exp Ther 314:139–147.

Miura K, Ishii T, Sugita Y, Bannai S (1992) Cystine uptake and glutathione levels are reduced by tetanus toxin. Brain Res 896:97–93.

Monery H, Choi DW (1989) Metabolism of extracellular glutamate in cortical cell cultures is reduced by leucine. Brain Res 446:144–148.

Monery H, Gifford RG, Hartley DM, Dugan LL, Goldberg MP, Choi DW (1992) Oxygen or glucose deprivation-induced neuronal injury in cortical cell cultures is reduced by leucine. Brain Res 896:97–93.

Monery H, Goldberg MP, Choi DW (1998) Glucose deprivation neuroinjury in cortical culture. Brain Res 483:347–354.

Moroni F, Attucci S, Cozzi A, Meil E, Picca R, Scheideler MA, Pellacciani R, Noe C, Sariecholu I, Pelegrini-Giampietro DE (2002) The novel and systemically active metabotropic glutamate 1 (mGlu1) receptor antagonist 3-MATIDA reduces post-ischemic neurological injury. Neuropharmacology 42:741–747.

Nellergd B, Wieloch T (1992) Cerebral protection by AMPA- and NMDA-receptor antagonists administered after severe ischemia-induced hypoglycemia. Exp Brain Res 92:259–266.

Novelli A, Reilly JA, Lysko PG, Hennebery RC (1988) Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res 451:205–212.

Patel SA, Warren BA, Rhoderick JF, Bridges RJ (2004) Differentiation of glioblastoma cells exposed to oxidative stress. Am J Pathol 162:250–258.

Piani D, Fontana A (1994) Involvement of the cystine transport system xCT in brain energy metabolism. J Cereb Blood Flow Metab 14:1–8.

Pongbun-Pongsabutr P, Hynchal C, Buchfelder M, Eyupoglu IY (2008) Small interfering RNA-mediated xCT silencing in glioma cells inhibits neurodegeneration and alleviates brain edema. Nat Med 14:629–632.

Shih AF, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH (2003) Coordinate regulation of glutathione biosynthesis and release by Nrf-2 expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394–3406.

Silverstein FS, Simpson J, Gordon KE (1990) Hypoglycaemia alters striatal amino acid efflux in perirstral rats: an in vivo microdialysis study. Ann Neurol 28:516–521.

Sontheimer H (2008) A role for glutamate in growth and invasion of primary brain tumors. J Neurochem 105:287–295.

Steel R, Torrie J (1980) Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill Book Co., New York.

Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, Swanson RA (2007a) Astrocyte glutamate sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor 2P-316,818 [IR-R’S]-1-Chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-(1-phenylmethyl)-propyl]-1H-indole-2-carboxamid). J Pharmacol Exp Ther 321:45–50.

Suh SW, Hamby AM, Swanson RA (2007b) Hypoglycemia, brain energetics, and hypoglycemic neuronal death. Glia 55:1280–1286.

Swanson RA, Benington JH (1996) Astrocyte glucose metabolism under normal and pathological conditions in vitro. Dev Neurosci 18:515–521.

Swanson RA, Yu AC, Chan PH, Sharp FR (1990) Glutamate increases glycogen content and reduces glucose utilization in primary astrocyte culture. J Neurochem 54:480–496.

Tanaka J, Toku K, Zhang B, Ishikawa K, Sakakura M, Maeda N (1999) Astrocytes protect neuronal death induced by reactive oxygen and nitrogen species. Glia 28:85–96.

Tasker RC, Coyte JT, Vornov J (1992) The regional vulnerability to hypoglycaemia-induced neurotoxicity in organotypic hippocampal culture: protection by removal of tetrodotoxin or delayed MK-801. J Neurosci 12:4298–4308.

Taylor AL, Hewett SJ (2002) Potassium-activated glutamate release liberated by neuronal injury. J Neurochem 77:43881–43887.

Tracckley TF, Ulilasu TF, Hewett SJ (2001) SIN-1-induced cytotoxicity in mixed cortical cell culture: peroxynitrite-dependent and -independent induction of excitotoxic cell death. J Neurochem 72:445–455.

Ulilasu TF, Hewett SJ (2000) A microtiter trypan blue absorbance assay for the quantitative determination of excitotoxic neuronal injury in cell culture. J Neurosci Methods 100:157–163.

Vivitsy V, Thomas M, Ghorpade A, Gendelman HE, Banerjee R (2006) A functional transsulfuration pathway in the brain links to glutathione homeostasis. J Biol Chem 281:35785–35793.

Walls AB, Heimbach CM, Bouman SD, Schouboe A, Waagepetersen HS (2009) Robust glutamate shunt activity in astrocytes: effects of glutamatergic and adrenergic agents. Neuroscience 158:284–292.

Wang XF, Cynader MS (2000) Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem 74:1434–1442.

Warr D, Takahashi M, Atwell D (1999) Modulation of extracellular glutamate concentration in rat brain slices by cystine-glutamate exchange. J Physiol 514:783–793.

Welch H, Bannai S (1987) Induction of cystine transport activity in mouse peritoneal macrophages. J Exp Med 165:628–640.

Watkins J, Collingridge G (1994) Phenylglyoxal derivatives as antagonists of metabotropic glutamate receptors. Trends Pharmacol Sci 15:333–342.

Westergen I, Nystrom B, Hamberger A, Johansson BB (1995) Intracerebral dialysis and the blood-brain barrier. J Neurochem 64:229–234.

Wieloch T (1985) Hypoglycaemia-induced neuronal damage prevented by an N-methyl-D-aspartate antagonist. Science 230:681–683.

Wieloch T, Engelsen B, Westerberg E, Auer R (1985) Lesions of the glutamatergic cortico-striatal projections in the rat ameliorate hypoglycaemic brain damage in the striatum. Neurosci Lett 58:25–30.

Xu C, Yagarstannam J, Lura R, Khoo CL, Ollai SS, Sim K (2011a) Persistent, severe hypoglycemia-induced organic brain syndrome with neurological sequelae: a case study. Gen Hosp Psychiatry 33:412.e9–412.e11.

Xu C, Yagarstannam J, Lura R, Khoo CL, Ollai SS, Sim K (2011b) Persistent, severe hypoglycemia-induced organic brain syndrome with neurological sequelae: a case report. Gen Hosp Psychiatry 33:412.e9–412.e11.

Ye ZC, Rothstein JD, Sontheimer H (1999) Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. J Neurosci 19:10767–10777.

Zhang Y, Barres BA (2010) Astrocyte heterogeneity: an undereappreciated topic in neurobiology. Curr Opin Neurobiol 20:588–594.