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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 *sut* 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 hypoglycemic 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

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Abbreviations: AraC, β-D-cytosine arabinofuranoside; BSS, balanced salt solution; CNS, central nervous system; CPG, carboxyphenylglycine; GD, glucose deprivation; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; NMDA, *N*-methyl-D-aspartate; qPCR, quantitative PCR; WT, wild-type.

demonstrated that astrocyte system x_c^- (cystine/glutamate antiporter) activity contributes to hypoxic neuronal death via a glutamate-mediated mechanism (Fogal et al., 2007; Jackman et al., 2010b). System x_c^- is a heteromeric amino acid transporter consisting of two subunits: xCT – 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 x_c^- is directly coupled to glutamate export, which occurs in a Na^+-independent manner (Bannai and Kitamura, 1980). Enhanced system x_c^- 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 x_c^- 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 x_c^- 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_0 (balanced salt solution) contains: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1 mM NaH_2PO_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_0 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_0 contained 100 μM 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 μM AraC (β-D-cytosine arabinofuranoside; Sigma Chemical Co.) once, for 5–6 days, to prevent microglia cell growth. WT (wild-type) and *sut* (xCT-deficient) astrocytes were cultured from cortices of single pups derived from *sut*/+ breeding pairs (JAX; Stock no. 001310). 2-Mercaptoethanol (55 μM) was added to the glial plating medium of *sut* cultures to support growth and to WT cultures for control purposes (Shih et al., 2006; Jackman et al., 2010b). The rest of the brain was used for genotyping: WT primers (230 bp product) 5′-GAAG-TGCTCCGTGAAGAAGG-3′ (forward), 5′-ATCTCAATCCTGG-GCAGATG-3′ (reverse); *sut* primers (≈2280 bp product) 5′-CCACTGTTGTAGGTCAGCTTAGG-3′ (forward), 5′-CAGG-ACCTGTGAATATGATAGGG-3′ (reverse). Purified astrocyte cultures were obtained by incubating astrocyte monolayers with 75 mM leucine methyl ester to remove any residual microglia as previously described (Hamby et al., 2006; Jackman et al., 2010b). At the time of experimentation, cultures were ≤35 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 μM AraC once for 2 days to prevent glial cell growth. The medium was partially replenished (½ 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 μl) into BSS_0. 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-aglycaemia 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 μM NMDA (*N*-methyl-D-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 x_c^- 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 ($\Delta\Delta C_T$) with β-actin as the housekeeping control as described (Jackman et al., 2010b). Importantly, β-actin C_T values were unaffected by IL-1β treatment. Amplification efficiency was >94%.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Software (Version 4.03) as described in each Figure legend. Because normalized data are, by nature, not normally distributed, percentage data were first transformed via arcsin square root (Steel and Torrie, 1980). Likewise, for qPCR, statistics were performed on the logarithmic retransformation (i.e. geometric means) of $2^{-\Delta\Delta CT}$ values. In all experiments, data are expressed as the means ± S.E.M. Significance was assessed at $P<0.05$.

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.

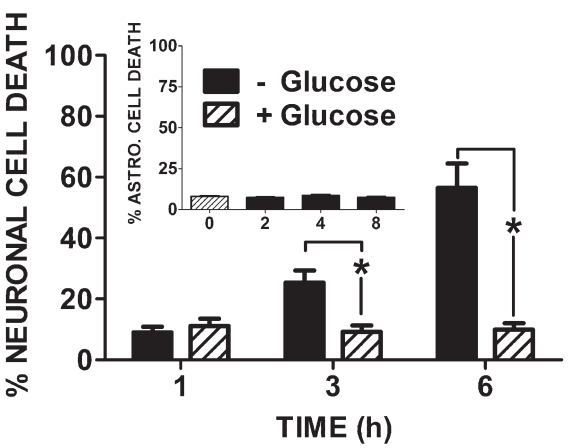

Figure 1 GD injures neurons, but not astrocytes *in vitro*

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).

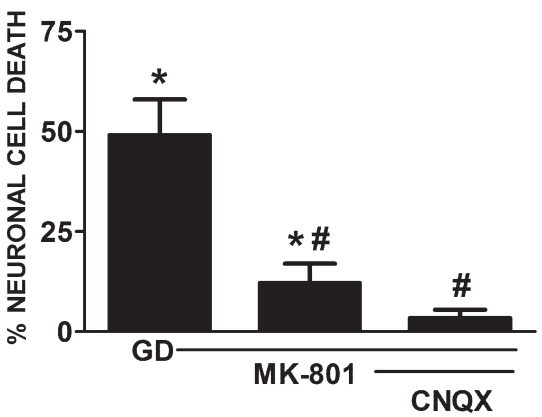

Figure 2 Hypoglycaemic neuronal cell death is attenuated by glutamate receptor antagonism

Mixed cortical cultures were washed into a BSS_0 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 BSS_0 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).

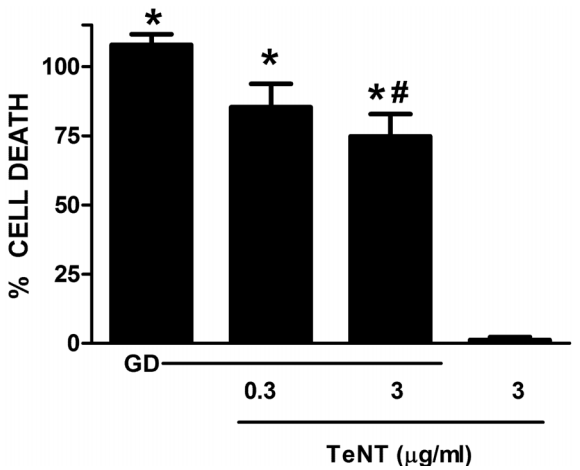

Figure 3 Inhibition of synaptic release of glutamate partially attenuates GD-induced neuronal cell death

Mixed cultures were incubated overnight with tetanus toxin (TeNT; 0.3–3 μg/ml) or its vehicle then washed into a BSS_0 (GD). Eight hour later, glucose (10 mM) was added and the cultures were placed back into the incubator, after which neuronal cell death was assessed 20–24 h later. NB: Values for GD indicate that all the neurons (100%) plus some of the astrocytes (7–8%) were killed. (*) Indicates values significantly different from control conditions ($=2.06\pm0.8\%$) determined by washing cells into BSS_0 followed by immediate addition of glucose, whereas (#) represents a significant diminution from GD-induced neuronal cell death as determined by one-way ANOVA followed by Student–Newman–Keul's *post-hoc* test ($n=3$).

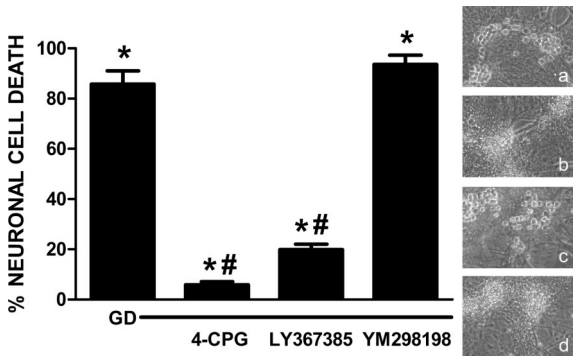

Figure 4 System x_c^- antagonism prevents neuronal cell death

Mixed cortical cultures were washed into BSS_0 in the presence or absence of the dual system x_c^-/mGluR1 antagonists 4-CPG (50 μM) and LY367385 (50 μM), or the selective mGluR1 inhibitor, YM298198 (10 μM). Eight hours later, supernatant was collected for measurement of neuronal cell death. (*) Indicates values that are significantly different from control conditions ($=7.98\pm0.89\%$) determined by washing cells into BSS_0 followed by immediate addition of glucose, while (#) represents a significant diminution from GD-induced neuronal death as assessed by one-way ANOVA followed by Student–Newman–Keul's *post-hoc* test ($n=11$–12 cultures from three independent experiments). Right: Representative phase micrographs of mixed cortical cultures: (a) control; (b) 8 h GD; (c) GD+4-CPG (50 μM); (d) GD+YM298198 (10 μM).

Overnight incubation of cultures with tetanus toxin prior to GD slightly, but significantly, attenuated neuronal damage in our system (Figure 3). More strikingly, addition of dual system x_c^-/mGluR1α antagonists [4-CPG (4-carboxyphenylglycine) or LY367385; 50 μM] (Gochenauer and Robinson, 2001) dramatically attenuated neuronal cell death resulting from GD (Figure 4). A role for mGluR1 is unlikely, given that the selective mGluR1 antagonist, YM298198 (10 μM), did not protect in this injury model. Importantly, all three inhibitors reduced dihydroxyphenylglycine (i.e. mGluR1)-mediated enhancement of hypoxic neuronal cell death at the concentrations utilized here (Fogal et al., 2007), demonstrating their effectiveness. These latter data suggest a role for system x_c^-.

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 can neither 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,

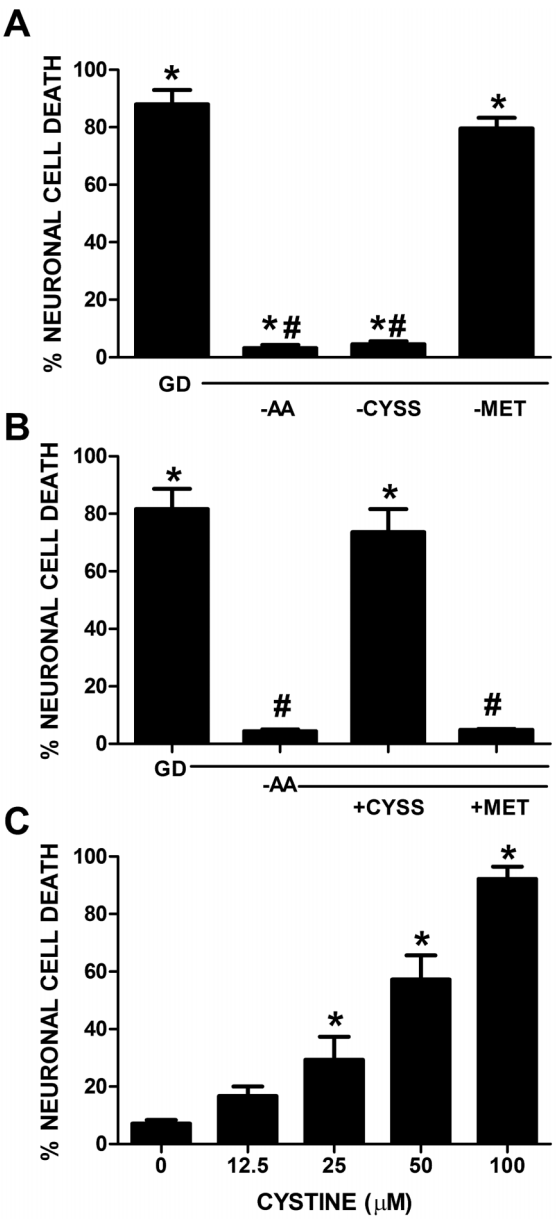

Figure 5 Hypoglycaemic neuronal death is dependent on L-cystine

(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 (=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 (=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 (=7.11 ± 1.27%) as determined by one-way ANOVA followed by Dunnett's *post-hoc* test (n=4).

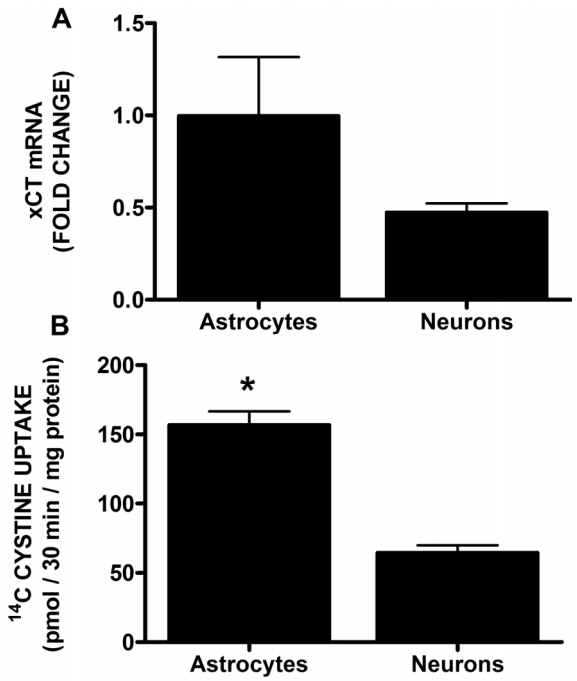

Figure 6 System x_c^- expression and activity is higher in astrocytes than in neurons

(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 ^{14}C-L-cystine (3 μM) for 30 min. Data are expressed as means ± S.E.M. ^{14}C-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 x_c^- 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; Monyer et al., 1989; 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

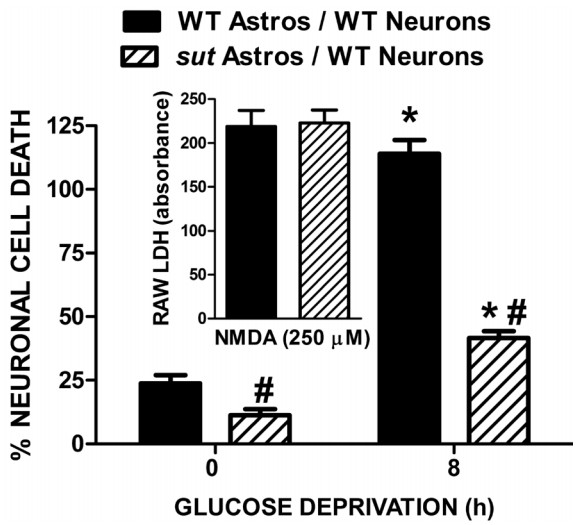

Figure 7 Hypoglycaemic neuronal death is dependent on astrocyte system x_c^-

Chimaeric cultures were obtained by plating WT neurons on astrocytes derived from *sut* mice (hatched white bars). These and control cultures (WT neurons on WT astrocytes; black bars) were washed into BSS_0, glucose added (final=10 mM) immediately to control cultures (0 h) or 8 h later to previously glucose-deprived cells (8 h), and neuronal cell death determined 20–24 h later. (*) Indicates a significant within-group difference, while (#) indicates a significant between-group difference as determined by two-way ANOVA followed by Bonferroni's *post-hoc* test (n=15–18 cultures from three independent experiments). Inset: LDH absorbance values for chimaeric and control cultures treated with 250 μM NMDA for 20–24 h.

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., 1998).

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 x_c^- (Figure 4) or via removal of its substrate, L-cystine (Figure 5). Although selective inhibitors for system x_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 x_c^-, which translocates glutamate into the extracellular space. While elimination of astrocytic system x_c^- is sufficient to prevent GD-induced neuronal death, we also found that enhancement of astrocytic system x_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 x_c^- pharmacological antagonism (Figure 8) argue for the primacy of system x_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 K_m of system x_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

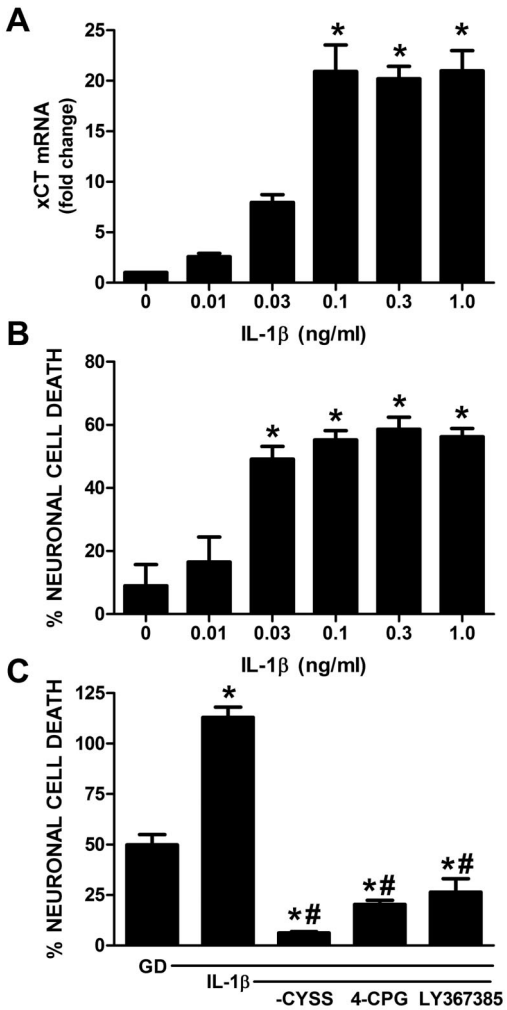

Figure 8 Enhanced astrocyte system x_c^- activity potentiates hypoglycaemic neuronal death

(**A**) Purified astrocytes (n=3 from three independent experiments) were incubated with IL-1β 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-1β for 20–24 h then washed into BSS_0. 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-1β) 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-1β (GD+IL-1β) or vehicle (GD) for 20–24 h, then washed into BSS_0 containing 4-CPG (50 μM) or LY367385 (50 μM) or one lacking cystine (−CYSS) for 4 h. (*) Indicates values different from GD. (#) Represents a significant diminution from the IL-1β-mediated potentiation of GD-induced neuronal death as determined by one-way ANOVA followed by Student–Newman–Keul's *post-hoc* test (n=6–16 cultures from four independent experiments).

x_c^- has no physiological role *in vivo*. Yet, numerous studies demonstrate that system x_c^- is an important contributor to the ambient extracellular glutamate levels that bathe the CNS *in vivo* (Jabaudon et al., 1999; Warr et al., 1999; Baker et al., 2002a, 2002b; Melendez et al., 2005; Augustin et al., 2007; Featherstone and Shippy, 2008; Massie et al., 2011; De Bundel et al., 2011) with maximal transporter activity estimated to theoretically increase extracellular glutamate by 0.6 μM/s (Cavelier et al., 2005). Additionally, direct comparison of measurement made in accumbens should not be extrapolated to cortex as differences in astrocyte biochemistry, physiology and function may be region specific (for reviews, see Hewett, 2009; Matyash and Kettenmann, 2010; Zhang and Barres, 2010). Further, insertion of a microdialysis probe can produce substantial tissue and vascular damage altering the accuracy of the measurements obtained (Westergren et al., 1995; Clapp-Lilly et al., 1999); and substantial technical difficulties have been reported when measuring cystine and cysteine concentrations (Bannai, 1984; Massie et al., 2011; De Bundel et al., 2011). Finally, it is important to note that plasma concentrations of cystine range from 5 to 60 μM (Brigham et al., 1960; Crawhall et al., 1968; Bannai, 1984; Eck et al., 1989; Wang and Cynader, 2000; Jones et al., 2002), which as stated above, is in line with the reported K_m value for cystine transport via system x_c^-. Due to the close apposition of astrocytes and blood vessels, cystine need only diffuse a small distance; thus it would not be surprising to find small concentrations in the extracellular space. This is reminiscent of the way excitatory amino acid transporters maintain low extracellular levels of glutamate with K_m values ranging from 1 to 100 μM (Danbolt, 2001). Further, despite the low levels of extracellular cystine measured in the accumbens, Baker and co-workers convincingly demonstrate that adaptations in system x_c^- underlie cocaine relapse in rat (Baker et al., 2003; Kau et al., 2008) and contribute to the psychotomimetic effects of phencyclidine (Baker et al., 2008).

Additionally, the activity of system x_c^- has been linked to excitotoxic neuronal cell death in a number of experimental paradigms *in vivo*. For instance, the export of glutamate via system x_c^- produces an excitotoxic necrosis that aids in glioma tumour growth and invasion (Savaskan et al., 2008; Sontheimer, 2008). Dopaminergic neuronal cell death induced by 6-hydroxydopamine is reduced in xCT knockout mice (Massie et al., 2011). While the contribution of system x_c^- has not been definitively characterized in cerebral ischaemic injury, systemic administration of CPG derivatives has been demonstrated to decrease the infarct volume after MCAO (middle cerebral artery occlusion) in rat (Moroni et al., 2002), a protective effect originally ascribed to inhibition of mGluR1. Yet, compared with their WT controls, neuronal injury following MCAO is not reduced in mGluR1-deficient mice (Ferraguti et al., 1997). Thus, inhibition of system x_c^- might underlie the protection of so-called mGluR1 antagonists in the aforementioned studies given their known ability to inhibit the system x_c^- transporter (Gochenauer and Robinson, 2001).

The fact that system x_c^- contributes to injury might seem paradoxical given its importance in the biosynthesis of the antioxidant molecule GSH (Watanabe and Bannai, 1987; Bannai et al., 1989; Miura et al., 1992; Sato et al., 1995; Bridges et al., 2001; Dun et al., 2006; Lewerenz et al., 2009). Ironically, while this pathway allows the CNS to rapidly up-regulate GSH in

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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