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Riluzole elevates GLT-1 activity and levels in striatal astrocytes

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Abstract

Drugs which upregulate astrocyte glutamate transport may be useful neuroprotective compounds by preventing excitotoxicity. We set up a new system to identify potential neuroprotective drugs which act through GLT-1. Primary mouse striatal astrocytes grown in the presence of the growth-factor supplement G5 express high levels of the functional glutamate transporter, GLT-1 (also known as EAAT2) as assessed by Western blotting and ³H-glutamate uptake assay, and levels decline following growth factor withdrawal. The GLT-1 transcriptional enhancer dexamethasone (0.1 or 1 μ M) was able to prevent loss of GLT-1 levels and activity following growth factor withdrawal. In contrast, ceftriaxone, a compound previously reported to enhance GLT-1 expression, failed to regulate GLT-1 in this system. The neuroprotective compound riluzole (100 μ M) upregulated GLT-1 levels and activity, through a mechanism that was not dependent on blockade of voltage-sensitive ion channels, since zonisimide (1 mM) did not regulate GLT-1. Finally, CDP-choline (10 μ M – 1 mM), a compound which promotes association of GLT-1/EAAT2 with lipid rafts was unable to prevent GLT-1 loss under these conditions. This observation extends the known pharmacological actions of riluzole, and suggests that this compound may exert its neuroprotective effects through an astrocyte-dependent mechanism.

Keywords

EAAT2; neuroprotection; citicholine; Parkinson's Disease; glutamate uptake; glutamate transporters

1. Introduction

Astrocytes are considered to have a key role in regulating neurodegenerative disease progression. One potentially important way of regulating glutamate levels, is the astrocyte glutamate transport system. Astrocytic glutamate transporters remove glutamate from the synaptic cleft and thus control the duration and magnitude of glutamate's actions (Beart and O'Shea, 2007; Danbolt, 2001). Down-regulation of the major transporter EAAT2 (known in rodents as GLT-1) is considered to be an important contributor to neurodegeneration and/or disease symptoms (Beart and O'Shea, 2007; Rattray and Bendotti, 2006).

In the last few years, a number of groups have identified clinically-useful drugs which are able to elevate GLT-1 levels in vitro (Boston-Howes et al., 2008; Colton et al., 2010; Ganel et al., 2006; Li et al., 2010; Rothstein et al., 2005). In the first study of this kind, Rothstein's

group reported that an orally-active beta-lactam antibiotic, ceftriaxone, elevates GLT-1 levels *in vitro*, and also has efficacy as a neuroprotectant in a transgenic mouse model of motor neurone disease (Rothstein et al., 2005).

We wish to identify drugs which act on astrocytes to elevate glutamate transporter levels that might afford symptomatic relief and neuroprotection in Parkinson's disease by reducing extracellular glutamate levels. As a first step towards this goal, we used striatal astrocytes in culture to test a number of orally active compounds in clinical use for their ability to up-regulate GLT-1 levels and activity. Compounds of different classes and mechanisms were chosen that have been previously suggested to increase astrocyte glutamate transporter function or attenuate neurodegeneration in other cellular systems or *in vivo*, namely ceftriaxone (Lee et al., 2008; Miller et al., 2008; Rothstein et al., 2005), riluzole (Azbill et al., 2000; Dunlop et al., 2003; Frizzo et al., 2004; Fumagalli et al., 2008), dexamethasone (Zschocke et al., 2005), zonisimide (Asanuma et al., 2010; Ueda et al., 2003) and CDP-choline (Hurtado et al., 2005; Hurtado et al., 2008).

2. Materials and Methods

2.1 Materials

Dexamethasone and riluzole hydrochloride were purchased from Tocris (Avonmouth, UK). Ceftriaxone sodium, citicoline sodium and zonisamide sodium salt and all other chemicals, unless specified otherwise were purchased from Sigma (Poole, UK). All cell culture reagents were obtained from Invitrogen (Paisley, UK).

2.2 Primary Culture of Mouse Astrocytes

Primary cultures of mouse striatal astrocytes were prepared from E15/E16 Swiss mouse embryos (NIH, Harlan, UK), a slight modification of previously reported methods (Bahia et al., 2008). Striata were dissected and gently dissociated by mechanical repetitive pipetting in phosphate-buffered saline (PBS, Ca^{2+} - and Mg^{2+} -free) supplemented with glucose (33 mM). Cells were plated into 6-well or 24-well Nunc multiwell plates that had been coated previously overnight with 1.5 $\mu\text{g/mL}$ poly L-ornithine (molecular weight 30,000-70,000) and then sequentially washed in water and PBS before coating with culture medium supplemented with 10% heat inactivated foetal bovine serum. Following removal of the final coating solution, cells were seeded ($9.5 \times 10^4/\text{cm}^2$) in culture media a medium composed of a mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (1:1 v/v) supplemented with 2 mM glutamine, 5 mM HEPES buffer (pH 7.4) and 10% heat inactivated fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5%. After 7 days *in vitro* (DIV) the culture medium was supplemented with the defined culture supplement G5 (insulin 5 $\mu\text{g/mL}$, transferrin 10 $\mu\text{g/mL}$, selenite 5.2 ng/mL, biotin 10 ng/mL, hydrocortisone 3.6 ng/mL, FGF2 5.2 ng/mL, EGF 10 ng/mL). After 4 DIV with G5 supplemented medium, cells were used for 3-day drug treatment in the G5 withdrawn growing medium when the astrocytes were confluent and no neurons could be found in the cultures by light microscopy. Cultures were also washed twice with PBS/glucose at 4 and 6 DIV to remove neuronal cells. For some experiments, cerebral cortical astrocytes were also prepared for comparative studies.

2.3 Immunocytochemistry of primary astrocytes

Cells grown on poly-L-ornithine coated 12mm round glass coverslips were fixed with 4% paraformaldehyde for 10 min. The cells were rinsed in PBS and then blocked and permeabilised with 0.2% Triton X-100/1% normal goat serum (NGS) in PBS and incubated overnight in an antibody cocktail containing 1% NGS and a mouse monoclonal GFAP antibody (1:400, Sigma) and rabbit anti GLT-1 antibody (Ab12 1:1000, gift of Dr D. Pow,

Brisbane, Australia) (Williams et al., 2005). The cells were rinsed in PBS and then incubated 1 h at RT in PBS/ 1% NGS containing secondary antibodies anti-mouse Alexa 488 (1:1000) and anti-rabbit Alexa 568 (1:1000) (Invitrogen, Paisley, UK). Nuclei were stained during 30 min with the nuclear dye Hoechst 33342 (1:1000) (Invitrogen, Paisley, UK). The coverslips were rinsed in PBS and mounted in an aqueous mounting media (Mowiol medium: 0.2 M Tris, pH 8.5, 1.6% Mowiol 40-88, 0.2% DABCO), 5% glycerol (v/v), 0.02% sodium azide). Control incubations leaving out the primary or secondary antibodies were performed for each antibody.

2.4 Western Blot Analysis

Cells grown in 6-well plates were rinsed with ice-cold PBS, pH 7.4, and scraped with lysis buffer (50 mM Tris, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml antipain, 2 µg/ml pepstatin A, 1 µg/ml chymostatin, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄ and 50 mM NaF). Lysates were collected and centrifuged at 2000 × g (5 min, 4 °C), after which protein concentration was determined using a Bradford protein assay (BioRad, Hemel Hempstead, UK). Protein samples (30 µg) were separated by 9% polyacrylamide gel electrophoresis and proteins transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare, Little Chalfont, U.K.) by semi-dry electroblotting. Membranes were blocked in TBS (20 mM Tris, pH 7.5, 0.5 M NaCl) containing 4% skimmed milk for 1 h. After washes with TBS containing 0.05% Tween-20 (TTBS), the membranes were incubated with the following antibodies in TTBS containing 1% skimmed milk powder: rabbit anti-GLT-1 (1/4000, gift of D. Pow, Brisbane), rabbit anti-GLAST (Anti A522 1/5000, gift of Dr N. Danbolt, Oslo) (Lehre et al., 1995), and rabbit anti-GAPDH (1/2000, Calbiochem International, Merck Chemicals Ltd, Nottingham, UK). Subsequently, the antigen-antibody complex was detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1/1000; Sigma, Poole, UK). Immunoreactive proteins were detected using ECL Western blotting detection reagents and autoradiography (GE Healthcare, Little Chalfont, U.K.). Bands were analysed using ImageJ (NIH, Bethesda, MD, USA). GLT-1 quantification was obtained by obtaining densities for both the 70 KDa lower (monomeric) band and the higher molecular mass ca. 200 KDa(multimeric) bands, which were then combined, as described previously (Suchak et al., 2003). In all cases, GLT-1 and GLAST band densities were normalised to the housekeeping protein control before combination for analysis. Data were analysed by One-Way ANOVA followed by Bonferroni's multiple comparison tests, where appropriate (Prism, GraphPad, La Jolla, USA). None of the drug treatments altered the levels of GAPDH protein.

2.5 Measurement of L-[³H]-glutamate transport

Uptake was carried out according to previously reported methods (Peacey et al., 2009). Astrocyte cultures in 24-well plates were incubated with radiolabeled glutamate (L-[³H] Glutamic acid, 20 nM, Amersham Biosciences) and unlabeled glutamate (Sigma) mixed to obtain a final concentration of 100µM in uptake buffer (5mM Tris, 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl₂, 1.2mM MgCl₂, 1.2 mM K₂HPO₄, 10 mM Glucose and 10 mM HEPES, pH 7.4). In each experiment, Na⁺-dependent transport was estimated by subtracting the data obtained by replacing sodium chloride with choline chloride. Uptake was determined after 5 min by removing the radioactive solution and rinsing with ice-cold choline chloride-containing Tris buffer (pH7.4). Cells were lysed in 0.1M NaOH, and the amount of the incorporated glutamate was determined by liquid scintillation counting of the cell lysate. When required, cells were incubated with the non-selective glutamate transporter inhibitor Threo-beta-benzyloxyaspartate (TBOA, Tocris Cookson, Avonmouth, UK. 1 mM) or the GLT-1 inhibitor WAY-213613 (gift of Dr. J. Dunlop, Wyeth, Princeton USA, 10 µM) at 37°C for 15 min before adding the substrate and during the glutamate uptake. Data were analysed by One-Way ANOVA followed by Bonferroni's multiple comparison tests, where

appropriate (Prism, GraphPad, La Jolla, USA). All experiments were carried out at least in triplicate, each experiment contained replicates from 2-3 culture wells.

3. Results

3.1 Striatal astrocytes in vitro possess GLT-1 levels and activity

First we established cell culture conditions to support glutamate transporter expression in striatal astrocytes. E15/16 mouse striatal astrocytes that are grown in astrocyte growth media (DMEM/Hams F12 containing 10% FCS) have little expression of the GLT-1 glutamate transporter after 10-12 days in culture (Fig 1a). Since GLT-1 expression in astrocytes is known to be highly dependent on growth factors (Figiel et al., 2003; Gegelashvili et al., 1997; Vermeiren et al., 2005), we therefore developed a protocol based on the observations of Vermeiren *et al.* (2005). After 7 days in vitro (DIV) in astrocyte growth medium, neurons are removed by dislocation and the culture medium is replaced with G5-supplemented astrocyte growth medium. Fig. 1 shows that after 7 DIV in astrocyte growth medium followed by 7 DIV in G5 supplemented astrocyte growth medium (G5 supplemented), the GLT-1 expression is detectable, and much higher than found in striatal astrocytes that are grown for 14 DIV in unsupplemented astrocyte growth medium (i.e. without G5; Fig. 1a, 1b), although not as high as typically found in cortical astrocytes (Fig 1c).

Western blotting confirms that there is a much higher level of GLT-1 expression in G5 supplemented astrocytes compared to astrocytes grown in unsupplemented growth medium (Fig. 1d, compare first two lanes), with levels of expression in striatal astrocytes were lower than found in cortical astrocytes (Fig 1d). Fig 1e shows quantification of GLT-1 band densities derived from Western blots from cortical or striatal astrocytes. For both types of astrocytes, very little GLT-1 was detectable on Western Blots when grown for 14 days in unsupplemented astrocyte growth medium. When cortical or striatal astrocytes were supplemented with G5, there was robust GLT-1 expression, G5 supplemented striatal astrocytes had approximately 45% lower GLT-1 levels than G5 supplemented cortical astrocytes ($n = 2$). If astrocytes were grown for 7 DIV in astrocyte growth medium followed by 4 DIV in G5 supplemented astrocyte growth medium, followed by 3 DIV in astrocyte growth medium alone (G5 withdrawal), approximately two-thirds of GLT-1 protein was lost, with the loss similar for cortical and striatal astrocyte cultures). The GLT-1 expression in cultures grown with G5 supplementation was, on average $264 \pm 3.5\%$ ($n = 6$) compared to G5 withdrawal cultures. We interpret the reduction in GLT-1 between G5 supplemented and G5 withdrawn cultures as a loss of GLT-1 protein rather than an incomplete induction GLT-1 gene expression, since in pilot experiments using cortical astrocytes, we found that the GLT-1 levels found after 7 days supplementation with G5 were no higher than found after 4 days supplementation with G5 (data not shown).

Pharmacological analysis (Fig. 1f) shows that the ^3H -glutamate uptake in G5 supplemented striatal astrocytes is completely blocked by replacing sodium with choline (inhibition = $99.3 \pm 0.3\%$, $n = 3$), and mostly blocked by the non-specific glutamate transporter inhibitor DL-TBOA (inhibition of $88.4 \pm 2.7\%$, $n = 4$), indicating that the bulk of glutamate uptake is through sodium-dependent glutamate transporters of the EAAT family. The GLT-1/EAAT2 selective inhibitor WAY-213613 blocks about 40% of ^3H -glutamate uptake ($38.7 \pm 14.3\%$, $n = 4$), and about half of the uptake is blocked by the GLAST/EAAT1 preferring inhibitor Serine- α -sulphate ($54.4 \pm 4.3\%$, $n = 4$) showing that both GLT-1 and GLAST contribute to glutamate uptake, as expected from studies of cortical astrocyte cultures grown under similar conditions e.g. Tortarolo et al. (2004). We note, however, that the relative proportion of uptake due to GLT-1 varied somewhat between cultures. Consistent with Western blot data, G5 supplemented striatal astrocytes supported a greater level of glutamate uptake activity compared to G5 withdrawal cultures (Fig. 1g).

3.2 Growth factor withdrawal provides a test-bed for discovery of compounds which can support GLT-1 levels

In order to identify compounds that enhance GLT-1 levels and activity *in vitro*, we grew primary striatal astrocytes for 7 days in astrocyte growth media, removed neurons and transferred to media containing G5 supplement for four days then into astrocyte growth media in the presence or absence of test compounds for a further 3 days before analysis by Western Blotting and ^3H -glutamate uptake assay. We reasoned that agents which either block GLT-1 down-regulation or enhance GLT-1 expression would be identified in these experiments, for their ability to increase GLT-1 protein levels and glutamate uptake activity.

Dexamethasone has previously been shown to increase GLT-1 in cortical astrocytes by increasing GLT-1 gene expression (Zschocke et al., 2005). In striatal astrocytes, dexamethasone administered to G5 withdrawn astrocytes at 0.1 μM or 1 μM for 3 days caused an increase in GLT-1 levels compared to vehicle administration, as determined by Western Blotting. A representative Western blot is shown in Figure 2a. Quantification revealed that the levels of GLT-1 in dexamethasone treated cultures were $275 \pm 38\%$ ($n = 6$, $p < 0.001$ for 0.1 μM dexamethasone and $205 \pm 35\%$ ($n = 5$, $p < 0.01$) for 1 μM dexamethasone, compared to G5 withdrawn control values (Fig. 2b). This degree of change shows that dexamethasone restores GLT-1 protein levels to similar levels found in astrocytes maintained in G5 medium. The levels of the other astrocyte glutamate transporter, GLAST (Fig. 2b). Astrocytes treated with 1 μM dexamethasone also showed an increase in sodium-dependent ^3H -glutamate uptake (Fig. 3a). Dexamethasone increased total glutamate uptake activity in striatal astrocytes by $128 \pm 6\%$ ($n = 9$, $p < 0.01$), compared to vehicle-treated controls to (Fig. 3a, left panel). Since total uptake is due to both GLT-1 and GLAST activity, we used the selective GLT-1/EAAT2 inhibitor WAY-213613 to identify the component of uptake accounted for by GLT-1 alone. GLT-1 activity was increased to $193 \pm 28\%$ of vehicle control values ($n = 9$, $p < 0.05$). Use of this inhibitor confirmed that almost all (78%) of the increase in glutamate uptake induced by dexamethasone was accounted for by increased GLT-1 activity. A lower concentration of dexamethasone (0.1 μM) had a similar effect on glutamate uptake activity (results not shown). Thus it is GLT-1 but not GLAST protein and activity levels which are upregulated by dexamethasone in these cultures.

3.3 Riluzole upregulates GLT-1 levels and activity in striatal astrocytes following growth factor withdrawal

Riluzole (100 μM) was administered for 3 days to striatal cultures in the three day period following G5 supplement withdrawal. Representative Western blots are shown in Figure 2a. Quantification of the data reveals that riluzole causes a significant increase in GLT-1 protein levels to $199 \pm 46\%$ of untreated control values ($n = 10$, $p < 0.01$) (Fig 2d). This degree of change suggests that riluzole increase GLT-1 protein levels about 75% of the levels found in astrocytes maintained in G5 medium. Riluzole treatment did not significantly alter the levels of GLAST protein, though a non-significant increase of $165 \pm 31\%$ compared to untreated control values was observed ($n = 10$, Fig 2d). Riluzole (100 μM) caused an increase in glutamate uptake to $138 \pm 8\%$ compared to vehicle-treated controls ($n = 9$, $p < 0.001$) (Fig 3b). Use of the GLT-1 selective inhibitor WAY213613 showed that an increase in GLT-1 function was to $140 \pm 13\%$ of control ($n = 9$, $p < 0.05$) (Fig 4b). Increased GLT-1 activity accounts for most (64%) of the increase in glutamate transport activity in these cultures.

3.4 The sodium channel blocker, zonisamide does not prevent loss of GLT-1 levels and activity following growth factor withdrawal

In preliminary experiments, zonisamide (10 nM – 1 mM) was tested for its ability to enhance GLT-1 levels in striatal astrocytes following G5 supplement withdrawal. As shown for the highest concentration tested (1 mM), zonisamide did not cause a change in levels of

GLT-1 or GLAST protein (Fig 2d), and failed to regulate either total ^3H -glutamate uptake or ^3H -glutamate uptake through GLT-1 (Fig 3c).

3.5 Ceftriaxone and CDP-choline fail to protect GLT-1 levels and activity from growth factor withdrawal in striatal astrocytes

We tested the ability of ceftriaxone (100 μM and 1 mM) to prevent or reverse the loss of GLT-1 following G5 supplement withdrawal. Western blotting revealed that ceftriaxone did not reverse the loss of GLT-1 protein (Fig 2a), indeed the levels of GLT-1 protein fell slightly, compared to vehicle-treated controls at the highest concentration of ceftriaxone tested, 1 mM though the decrease was not statistically significant (Fig 2e). GLAST protein was also unaffected by ceftriaxone (Fig 2e). The ability of ceftriaxone (10 μM - 1 mM) to regulate ^3H -glutamate uptake was also tested (Fig 4a), and at the highest concentration used, glutamate transport was slightly, but significantly down-regulated to $85 \pm 7\%$ of vehicle-treated control levels ($n = 6$, $p < 0.01$).

We tested the ability of CDP-choline (10 μM - 1 mM) to prevent or reverse the loss of GLT-1 levels and activity caused by withdrawal of G5 supplement. Western blotting (Fig 2a) revealed that CDP-choline at each of the concentrations used did not regulate GLT-1 or GLAST protein levels (Fig 2f) or total ^3H -glutamate uptake activity (Fig 4b).

Discussion

Glutamate transporters expressed in astrocytes are critical for maintaining the extracellular concentration of glutamate below toxic levels in the central nervous system (Beart and O'Shea, 2007; Danbolt, 2001). Since astrocytes are preserved during neurodegeneration, they represent bonafide targets for neuroprotective drug treatment. In recent years, a number of clinically useful drugs have been shown, using a variety of *in vitro* and *ex vivo* model systems to increase the glutamate uptake capacity of astrocytes. In this study we have focussed on a functional endpoint, namely GLT-1 protein levels and activity, and have shown for the first time that riluzole is able to increase astrocyte GLT-1 levels and activity following growth factor withdrawal.

Because of our specific interest in anti-parkinsonian therapies, we developed a system to monitor GLT-1 levels and activity in astrocytes from the striatum, a region known to receive elevated glutamate transmission in Parkinson's disease patients and animal models (Anglade et al., 1996; Calabresi et al., 1993; Chassain et al., 2005; Lindfors and Ungerstedt, 1990). To our knowledge, there are few published descriptions of glutamate uptake into striatal astrocytes in culture (Schluter et al., 2002). Since astrocytes from different brain regions have diverse neurochemical properties (Wilkin et al., 1990), we adapted published protocols (Vermeiren et al., 2005) to grow striatal astrocytes so that they maintained a good level of GLT-1. Even under optimal conditions, our data show that striatal astrocytes support less GLT-1 expression than cortical astrocytes, suggesting that the striatum is less able to handle elevated glutamate levels than other CNS regions.

To test compounds for their ability to upregulate GLT-1, we designed our assay system to mimic an aspect of neurodegeneration namely GLT-1 down-regulation following denervation (Levy et al., 1995) or growth factor withdrawal (Figiel et al., 2003; Gegelashvili et al., 1997; Vermeiren et al., 2005). As expected, following growth factor withdrawal in striatal astrocytes there is a substantial loss of astrocyte GLT-1 over a three day period. Drug-induced increases in GLT-1 following growth factor withdrawal may be through a variety of mechanisms including increasing mRNA levels, increased protein expression and/or reducing GLT-1 protein turnover. In this study we chose to screen five compounds of different classes and mechanisms that have been previously suggested to increase astrocyte

glutamate transporter function and/or attenuate neurodegeneration *in vitro* and/or *in vivo*. Three of the compounds we chose have been reported to be transcriptional enhancers of GLT-1 in other systems, namely dexamethasone, ceftriaxone and riluzole (Lee et al., 2008; Liu et al., 2011; Zschocke et al., 2005). The pharmacological activities of zonisamide and riluzole are usually ascribed to a blockade of voltage-sensitive ion channels (Bellingham, 2011; Leppik, 2004), while CDP-choline is proposed to act by increasing the association of the glutamate transporter with lipid rafts (Hurtado et al., 2008).

Dexamethasone has been previously shown to be an efficient inducer of GLT-1 in cortical astrocytes *in vitro*, via a glucocorticoid receptor-dependent mechanism which induces GLT-1 mRNA levels (Zschocke et al., 2005). Our data confirmed that, as for cortical astrocytes, the corticosteroid, dexamethasone, caused an upregulation in GLT-1 levels with a concomitant increase in GLT-1 activity. These results confirm that the striatal astrocyte culture system is sensitive to the effect of a known GLT-1 transcriptional inducer.

The beta-lactam antibiotic, ceftriaxone, was identified from a screen of FDA approved compounds to upregulate GLT-1 in organotypic cultures, and to confer neuroprotection in mice (Rothstein et al., 2005). Ceftriaxone has been reported to upregulate GLT-1 in primary human astrocytes via enhancing GLT-1 gene transcription (Lee et al., 2008). In our system, ceftriaxone at a range of concentrations did not cause an increase in GLT-1 protein levels, nor regulated glutamate uptake activity, suggesting that the ability of ceftriaxone to induce GLT-1 was not sufficient to overcome the GLT-1 loss consequent to growth factor withdrawal. While there is a body of evidence for ceftriaxone neuroprotection *in vivo*, and these effects are assumed to be consequent to upregulation of GLT-1 (Ramos et al., 2010; Rothstein et al., 2005; Verma et al., 2010), we note that not all studies have been able to confirm that GLT-1 can be upregulated by ceftriaxone *in vivo*. Indeed, recent evidence suggests that the way in which ceftriaxone is neuroprotective *in vivo* is through upregulation of an antioxidant defence system, involving the glutamate:cystine exchanger system (Lewerenz et al., 2009). Here we have used a system of growth factor withdrawal to drive down-regulation of GLT-1, which we believe is a good model system to assess astrocyte function in neurodegenerative disease states. That ceftriaxone is unable to overcome GLT-1 down-regulation in this system suggests that its ability to induce GLT-1 gene expression is weak, compared to other compounds such as dexamethasone. Our data shows also that the positive effects of drugs on glutamate transporter expression is dependent on the exact system and experimental conditions used, and highlights the need to replicate positive findings in multiple assay systems.

Riluzole is a neuroprotective compound licensed for clinical use in Amyotrophic Lateral Sclerosis (ALS) with a modest but proven efficacy that, on average, extends lifespan of people with ALS by 7 months (Bensimon et al., 1994). Here we show, for the first time, that riluzole selectively regulates GLT-1 levels and activity after growth factor withdrawal from striatal astrocytes. Upregulation of GLT-1 levels is not a non-specific response of astrocytes to drug treatment, since another abundant transporter expressed in astrocytes, GLAST, is not regulated by riluzole (or dexamethasone). The observation of GLT-1 increase is supported by a recent report using reporter gene expression in a cell line which showed that riluzole enhances EAAT2 gene expression (Liu et al., 2011). Riluzole has already been shown to acutely modulate GLT-1/EAAT2 activity. A number of independent studies have shown that riluzole activates glutamate uptake in cortical astrocytes, synaptosomes from spinal cord (which contain resealed astrocytic membranes, (Hirst et al., 1998; Suchak et al., 2003) or cell-lines expressing recombinant excitatory amino acid transporters (EAATs) (Azbill et al., 2000; Dunlop et al., 2003; Frizzo et al., 2004; Fumagalli et al., 2008).

Riluzole is regarded as a glutamate release inhibitor (Martin et al., 1993; Pratt et al., 1992) with riluzole's actions at therapeutically useful concentrations correlating with the drug's ability to inhibit sodium currents and reduction of repetitive firing as explained by Bellingham (2011) in his recent systematic review. To address whether this primary mechanism can account for GLT-1 induction, we examined whether another voltage-gated ion channel blocker, zonisamide (Leppik, 2004) could elicit a similar response. Since zonisamide was ineffective in regulating GLT-1, we conclude that the effect of riluzole is not dependent on a mechanism consequent to voltage-gated ion channels. Indeed, our observation adds to the range of effects of riluzole which cannot easily be accounted for by its effect on voltage-sensitive sodium channels. For example riluzole and its derivatives have antioxidant properties (Anzini et al., 2010), which may contribute to neuroprotection. Benzothiazoles, including riluzole have been shown to reduce protein aggregation (Heiser et al., 2002). Riluzole has been proposed to inhibit Protein Kinase C (Noh et al., 2000). Recently, in cell lines and neurons, riluzole has been shown to increase the activity of the cytoprotective transcription factor HSF-1, through inhibiting its degradation (Liu et al., 2011; Yang et al., 2008), though the primary mechanism by which this occurs is unknown. Riluzole can, through unknown mechanisms, activate Wnt signaling in cells (Biechele et al., 2010), and enhance neurofilament mediated-transport in neurons (Stevenson et al., 2009). Riluzole has long been known to modulate astrocytes so that they might confer neuroprotection to neurons (Peluffo et al., 1997). Regulation of glutamate transporter expression in astrocytes, adds to the list of effects which riluzole exerts on astrocytes which include suppression of astrocyte reactivity including inhibition of swelling- induced chloride channels (Bausch and Roy, 1996) and suppression of GFAP production *in vivo* (Carbone, Duty & Rattray, unpublished observations). Riluzole can induce the production of neurotrophins GDNF, NGF and BDNF in astrocytes (Caumont et al., 2006; Mizuta et al., 2001; Tsuchioka et al., 2011). The exact mechanism by which riluzole exerts its effects to promote GLT-1 expression is not known and subject to further investigation in our laboratories.

Another drug which failed to regulate GLT-1 levels and activity in our hands was CDP-choline, a dietary supplement with a wide range of proposed health benefits. CDP-choline has been shown to enhance EAAT2 activity in cultured rat astrocytes by increasing the association of GLT-1 with lipid rafts and via this mechanism has been proposed to partly underlie its beneficial effect in animal models of stroke (Hurtado et al., 2005; Hurtado et al., 2008). Here we show that CDP-choline is unable to preserve or upregulate GLT-1 from growth factor withdrawal.

There is a body of evidence that modulating the glutamate system may be a useful therapeutic strategy in neurodegenerative diseases. The current study highlights the importance of cell-based testing in a range of model systems to identify those compounds with the most promise to take forward into preclinical models, and clinical trials. Here we have identified riluzole as a compound which produces robust and selective increases in GLT-1 levels and activity in striatal astrocytes. This raises the intriguing possibility that riluzole may exert its neuroprotective effects *in vivo* via an astrocyte-dependent mechanism leading to enhanced glutamate reuptake, rather than by regulating glutamate release. These data have relevance to the potential treatment of Parkinson's disease.

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Abbreviations used

ALS	amyotrophic lateral sclerosis
BDNF	brain derived neurotrophic factor
CDP choline	cytidine 5' diphosphocholine
DIV	days in vitro
EAAT2	Excitatory amino acid transporter 2
EGF	Epidermal Growth Factor
FGF2	Fibroblast Growth Factor 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate and aspartate transporter
GLT-1	glutamate transporter 1
NGF	nerve growth factor
NGS	normal goat serum
TBOA	^{DL} -Threo-beta-benzyloxyaspartate

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Fig. 1.

(a-c) Representative immunofluorescent photomicrographs showing effect of G5 supplementation on GLT-1 expression in striatal astrocyte cultures grown for 14 DIV in astrocyte medium (a) or for 7DIV followed by 7 days in presence of G5 supplement (b). Cortical astrocytes supplemented with G5 medium are shown for comparison (c). Cultures were stained for GFAP (red) and GLT-1 (green) with nuclei counter-stained with Hoescht3342 (blue). (d) shows Western blotting of striatal astrocyte (SA) in astrocyte medium without G5 supplementation (-) or with G5 supplementation (+). Cortical astrocytes supplemented with G5 (CA) and cortical neurons (CN) are shown for comparison. Samples were probed with an anti-GLT-1 antibody, revealing monomeric bands at c. 67 KDa (lower arrow) and multimeric bands at ca. 90 KDa (upper arrow). The same blots probed with β -tubulin are shown (F, lower panel), revealing an absence of neurons in the primary astrocyte cultures. (e) Shows quantification of GLT-1 bands from cortical or striatal astrocytes grown for 14DIV in astrocyte medium without G5 supplementation (no G5), with 7 days G5 supplementation or with 4 days G5 supplementation followed by 3 days without G5 (G5 withdrawal). Graph shows mean density of monomeric and multimeric GLT-1 bands $\pm$ SEM (n = 3-5). (f) Graph shows ^3H -glutamate uptake activity in G5-supplemented striatal astrocyte cultures, and the effect of incubation in sodium-free buffer or with glutamate uptake inhibitors DL-Threo-beta-benzyloxyaspartate (DL-TBOA, 1 mM), WAY-213613 (WAY613, 10 μM), Serine-o-sulphate (SOS, 1 mM). Values show ^3H -glutamate uptake $\pm$ SEM expressed as % of maximal uptake in the absence of inhibitors (n = 4). (g) shows saturation curves for ^3H -glutamate uptake into striatal cultures in cultures which were G5 supplemented (solid line) or under G5 withdrawal (dashed line). Mean ^3H -glutamate uptake is plotted $\pm$ SEM (n = 3),

Fig. 2.

(a) shows representative Western blots from primary astrocyte cultures grown for 10 DIV followed by 4 days of G5 supplementation then a further 3 days in the absence (Con) or presence of drugs as follows: Dex = 1 μ M dexamethasone; Ril = 100 μ M riluzole; Zon = 1 mM zonisamide; cef = 1 mM ceftriaxone; CC = 1 mM CDP-choline. Blots were probed with antisera against GLT-1 (top panel), GLAST (middle panel) and a housekeeping protein GAPDH (bottom panel). GLT-1 and GLAST exist as monomeric (ca. 67 KDa) and trimeric (ca. 190 KDa) species, GAPDH is ca. 40 KDa. (b – e) GLT-1 and GLAST band intensities from blots were quantified, normalized to the housekeeping control, and plotted as relative band intensities $\pm$ SEM. Graphs show GLT-1 (black bars) and GLAST (grey bars) in comparison to G5-withdrawal controls for (b) dexamethasone (n = 10), (c) riluzole (n = 10), (d) zonisamide (n = 5), (e) ceftriaxone (n = 4) and (f) CDP-choline (n = 5). Analysis was carried out using an unpaired t-test (dexamethasone, riluzole, zonisamide) or One-way ANOVA (ceftriaxone, CDP-choline). *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Fig. 3.

The effect (a) 0.1 μM dexamethasone ($n = 9$), (b) 100 μM riluzole ($n = 9$) or (c) 1 mM zonisamide ($n = 6$) on total ^3H -glutamate uptake (unfilled columns) and the GLT-1 specific component of uptake (filled columns). Mean values for ^3H -glutamate uptake (pmol/min/mg protein) are shown $\pm$ SEM. * = $p < 0.05$ compared to vehicle control columns (One Way ANOVA followed by Bonferroni's multiple comparison test).

Fig 4.

The effect of (a) ceftriaxone ($n = 4$), (b) CDP-choline ($n = 4$) on total ^3H -glutamate uptake. Mean values for ^3H -glutamate uptake (pmol/min/mg protein) are shown $\pm$ SEM. * = $p < 0.05$ compared to vehicle control columns (One Way ANOVA followed by Bonferroni's multiple comparison test).