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**Links between L-glutamate transporters, Na⁺/K⁺-ATPase and cytoskeleton
in astrocytes: evidence following inhibition with rottlerin.**

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Key words:

glutamate transporters; astrocytes; rottlerin; cytoskeleton; trafficking; Na⁺/K⁺-ATPase

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Abstract

Astrocytes are plastic cells that play key roles in brain physiology and pathology, including via their glutamate transporters, EAAT1 and EAAT2, maintaining low extracellular glutamate concentrations and protecting against excitotoxic neuronal injury. Alterations in cell surface expression of EAATs and astrocytic cytoskeleton are important for regulating transporter activity. This study employed the actions of rottlerin, to interrogate the regulation of EAAT activity, expression and localization, and interfaces with Na⁺/K⁺-ATPase and astrocytic morphology. EAAT activity and expression were determined in primary cultures of mouse astrocytes in the presence of and after rottlerin removal, with or without trafficking inhibitors, using uptake ([³H]D-aspartate, ⁸⁶Rb⁺) and molecular analyses. Astrocytic morphology and EAAT localization were investigated using Western blotting and immunocytochemistry, in concert with image analysis of GFAP, F-actin and EAAT1/2. Rottlerin induced a time-dependent inhibition of glutamate transport ($V_{\max}$). Rapid changes in cytoskeletal arrangement were observed and immunoblotting revealed increases in EAAT2 total and cell surface expression, despite reduced EAAT activity. Rottlerin-induced inhibition was reversible and its rate was increased by monensin co-treatment. Rottlerin inhibited, while monensin stimulated Na⁺/K⁺-ATPase. Removal of rottlerin rapidly elevated Na⁺/K⁺-ATPase activity beyond control levels, while co-treatment with monensin failed to stimulate the Na⁺/K⁺-ATPase. These data reveal inhibition of EAAT activity by rottlerin is not associated with loss of EAATs at the cell surface, but rather linked to cytoskeletal rearrangement, and inhibition of the Na⁺/K⁺-ATPase. Rapid recovery of Na⁺/K⁺-ATPase activity, and subsequent restoration of glutamate uptake indicates that astrocytic morphology and EAAT activity are co-regulated by a tightly coupled, homeostatic relationship between Glu uptake, the electrochemical gradient and the activity of the Na⁺/K⁺-ATPase.

Key words: glutamate transporters; Na⁺/K⁺-ATPase; astrocytes; rottlerin; cytoskeleton; trafficking

Abbreviations

ATP, adenosine 5'-triphosphate; BFA, brefeldin A; CNS, central nervous system; D-Asp, D-Aspartate; DMSO, dimethyl sulphoxide; EAATs, excitatory amino acid transporters; GFAP, glial fibrillary acidic protein; Glu, L-Glutamate; MTT, 3-(4,5-dimethylthiazol2yl)-2,5-diphenyltetrazolium bromide; NDS, normal donkey serum; NGS, normal goat serum.

1.1 Introduction

L-Glutamate (Glu) is the major excitatory amino acid in the mammalian central nervous system (CNS), and the activation of Glu receptors by extracellular Glu is a tightly regulated process as high extracellular Glu concentrations lead to an excitotoxic injury cascade and neuronal injury (Beart and O'Shea, 2007). The concentration and half-life of Glu in the synapse is regulated by sodium-dependent uptake via excitatory amino acid transporters (EAATs), mostly astrocytic EAATs with EAAT2 and to a lesser extent EAAT1, responsible for the bulk of Glu uptake in the CNS (Rothstein et al., 1996, Tanaka et al., 1997). EAATs are vital to maintaining Glu homeostasis and EAAT dysfunction or loss of EAAT expression has been linked to various neurodegenerative diseases including amyotrophic lateral sclerosis, epilepsy, cerebral ischemia, Alzheimer's disease, Parkinson's disease and Huntington's disease (Beart and O'Shea, 2007, Sheldon and Robinson, 2007). While Glu uptake is energetically costly, EAATs are able to drive Glu uptake against the concentration gradient by utilizing the strong electrochemical gradient generated by the Na^+/K^+ -ATPase (Beart and O'Shea, 2007). Evidence of interactions between EAATs and the Na^+/K^+ -ATPase has begun to emerge (Rose et al., 2009) and recent molecular studies support the compartmentalization of these proteins (Genda et al., 2011, Bauer et al., 2012) in a macromolecular complex whose function is strongly dependent on energy demands.

Optimal EAAT cell surface expression is vital for Glu uptake and Glu homeostasis, particularly when extracellular Glu levels are high. In astrocytes, the majority of EAAT protein is expressed in the plasma membrane (>80% for EAAT2), with evidence for small intracellular pools of EAAT protein and trafficking of transporters (Susarla et al., 2004, Sheldon et al., 2006, Benediktsson et al., 2012). While there are a number of factors that are known to affect the regulation of EAAT expression at the cell surface, this area still remains

poorly understood. Protein trafficking is likely to be involved in the homeostatic regulation of EAAT activity, although it is difficult to discriminate between internalization, trafficking and subsequent changes in level of EAAT cell surface expression (Duan et al., 1999, Tai et al., 2007) versus changes in the catalytic efficiency of the transporter (Gonzalez et al., 2002, Adolph et al., 2007). EAAT activity and expression are regulated upon changes in astrocytic morphology. Astrocytes undergo substantial cytoskeletal reorganization both during development and in response to injury exemplified by astrocyte stellation and reactive astrogliosis (Ridet et al., 1997, Maragakis and Rothstein, 2006). Indeed, evidence for the strong relationship between astrocytic morphology and EAAT activity has recently begun to emerge with, in general a loss of EAAT2 and preservation of EAAT1 associated with reactive changes (Schlag et al., 1998, Bendotti et al., 2001, O'Shea et al., 2006, Lau et al., 2010, Lau et al., 2011, Lau et al., 2012).

In recent work we found that pharmacological treatments (e.g. Rho kinase inhibition) that led to alterations in astrocytic morphology elevated expression at the cell surface compartments and a consequent increase in transporter $V_{\max}$ (O'Shea et al., 2006, Lau et al., 2011). In this study we investigate the reverse event i.e. the response and recovery from Glu transporter inhibition. Rottlerin, a compound with a variety of effects including inhibition of PKC δ , was chosen since it has been shown to inhibit EAAT1 activity, reducing transport $V_{\max}$ following inducing EAAT internalisation and to reduce EAAT1 cell surface expression in astrocytic cultures (Susarla and Robinson, 2003). In this study we investigate the effect of Glu transport on astrocyte morphology and EAAT distribution. Using primary cultured mouse astrocytes expressing both EAAT1 and EAAT2 we report for the first time that in addition to inhibiting EAAT activity, rottlerin causes changes in EAAT distribution and cytoskeletal rearrangement. In addition we provide evidence that rottlerin inhibition is rapidly reversible

and enhanced by co-treatment with the Na^+ ionophore monensin. Furthermore, the mechanism of loss of EAAT activity and its recovery was not due to trafficking but rather rottlerin acting as a metabolic un-coupler affecting the function of the Na^+/K^+ -ATPase with consequent inhibition of EAAT activity. This study provides new evidence of the link between EAAT activity and astrocytic morphology and supports the importance of the inter-dependent relationship between Glu uptake and the Na^+/K^+ -ATPase (Danbolt, 2001, Beart and O'Shea, 2007, Sheldon and Robinson, 2007).

1.2 Experimental Procedure

1.2.1 Primary Astrocyte Cultures

Primary astrocytes were cultured from the brains of 1.5 d C57Bl6 mice as previously described (O'Shea et al., 2006). All experimentation received ethical approval and was undertaken according to the guidelines of the NHMRC (Australia). Briefly, forebrains of mice were removed, placed in ice cold HBSS solution and chemically digested in a trypsin solution with trituration using a 15 gauge needle. The homogenate was centrifuged and resultant pellet re-suspended in astrocytic medium (AM) prepared from DMEM (Gibco™ Invitrogen Corporation, Melbourne, Australia) containing 10% heat inactivated certified fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin and 0.25% (v/v) Fungizone™, preheated to 37°C at a volume of 5 ml per brain and plated at 10 ml per 75 cm² flask (NUNC, Copenhagen, Denmark). Cells were plated at 10 ml/75 cm² flask (2 brains/flask) and maintained in a humidified incubator supplied with 5% CO₂ at 37°C. Medium was changed twice weekly by aspiration and replacement with fresh pre-heated AM. Sub-culturing generated “secondary” cultures and cells were seeded onto plates with or without glass coverslips (1 x 10⁴ cells/cm²) at 10 *div*. A full media change was performed twice weekly with pre-heated AM to remove non-adherent cells. Drug treatments were performed when astrocytes became confluent at 22 *div* (12 *div* after subculturing). For all drug treatments, stock solutions were prepared in DMSO (Sigma-Aldrich, Sydney, Australia) and diluted using pre-heated AM maintained at a final volume of 0.5 ml/well. Drug concentrations were determined in preliminary dose response studies or from previous studies in the literature.

1.2.2 [³H]D-Aspartate Uptake

The activity of Glu transport was determined by monitoring the uptake of [³H]-D-Aspartate ([³H]-D-Asp) (Apricò et al., 2004). At the completion of drug treatments, cells were pre-

incubated at 37 °C for 5 min and then incubated with [³H]-D-Asp (Perkin-Elmer Life Sciences Inc, Boston, USA) in uptake buffer (50 nM) with or without unlabeled D-Asp (0.1 μM - 1 mM) or with L-Glu (1 mM, non-specific uptake) in uptake buffer. Uptake was terminated by washing at 4°C with Na⁺-free buffer and accumulated radioactivity determined using scintillation spectrometry. The rate of transport (V_{max}) and affinity (K_m) of EAATs were determined when [³H]-D-Asp uptake was performed in the presence of increasing concentrations of D-Asp (0.1 μM –1 mM) or Glu (1 mM). D-Asp uptake values were normalized against protein content. Data analysis was performed using GraphPad Prism v.5.0 (GraphPad, San Diego, USA).

1.2.3 Cell Viability

Cellular viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; an index of mitochondrial function; Sigma-Aldrich) reduction assay (Lau et al., 2011). Cells were incubated for 30 min at 37°C, MTT was aspirated, DMSO (Sigma-Aldrich) was added and cell viability determined colometrically (570 nm) on a Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, USA).

1.2.4 Immunocytochemistry

Cells on glass coverslips were fixed with paraformaldehyde (4%) in phosphate-buffered saline (PBS, pH 7.4), washed extensively and blocked in PBS containing 10% normal donkey serum (NDS) and 0.3% TX-100 at RT for 1 h. Cells were then washed rapidly with cold PBS, and incubated with selected primary antibodies (EAAT1 anti A522-541, 1:1500, Dr. Niels C. Danbolt, University of Oslo, Norway; EAAT2 anti B12-26, 1:1500, Dr. Niels C. Danbolt, University of Oslo, Norway and Dr. David V. Pow, RMIT University, Australia; GFAP 1:3000, (Chemicon, Melbourne, Australia); panCadherin 1:250 (Sapphire Biosciences,

Redfern, Australia) GM130 1:250 (Sapphire Biosciences) in 2% NDS with 0.3% TX-100, in PBS at 4°C overnight. The following day, cells were washed with PBS and incubated with appropriate secondary antibodies attached to Alexa Flour-conjugated antibodies (Molecular Probes Invitrogen, Melbourne, Australia) in 2% NDS with 0.3% TX-100, in PBS for 3 h. Staining for F-actin and Hoechst 33342 was carried out using rhodamine-conjugated phalloidin (1:3333; included in the incubation with secondary antibodies), diluted in 2% NDS in PBS containing 0.3% TX-100 and incubated for 3 h at room temperature. Coverslips were mounted on slides with Dako Fluorescent mounting media (Dako, Glostrup, Denmark). Fluorescence was visualized and digital images were acquired using an Olympus FluoView 1000 inverted confocal microscope with compatible software. Image analysis was performed using ImageJ software (obtained from the NIH website: <http://rsb.info.nih.gov/ij>) as described previously (Lau et al., 2011).

1.2.5 Biotinylation of Cell Surface Proteins, Protein Determination and Western blot

Analysis

These procedures have been described previously (O'Shea et al., 2006). Samples were pooled from 6 wells ($n = 3$ replicates) and total cell protein concentration determined with the Bio-Rad Dc Assay Kit (Sydney, Australia) according to the manufacturer's specifications. Western blots were performed where equal volumes of all three fractions (total, intracellular and cell surface) were loaded onto gels and membranes were incubated with primary antibodies (EAAT1 anti-A522, 1:15,000; GLT-1 1:10,000) at 4°C, overnight. The following day, membranes were washed and incubated with IRDye conjugated secondary antibodies (LiCor) and imaged using the Odyssey Classic near infrared detection system. Blots were quantified by taking the mean gray value of EAAT1 and EAAT2 bands normalised to β -actin level after subtracting background intensity.

1.2.6 Rubidium ($^{86}\text{Rb}^+$) Uptake

$^{86}\text{Rb}^+$ uptake into astrocytes was performed as previously described with minor adaptations (Aschner et al., 1990). At the completion of drug treatments, astrocytes were incubated with AM containing $^{86}\text{RbCl}$ (2 $\mu\text{Ci/ml}$; 4 μM) for 20 min at 37°C. After 20 min incubation the reaction was stopped by aspirating the medium and placing the cells on ice. Cells were then rinsed 3 times with cold buffer (choline chloride 150 mM, MgCl_2 1.2 mM, CaCl_2 1.2 mM, BaCl_2 2 mM, HEPES 5mM, osmolarity adjusted to 340 mOsm with mannitol and pH adjusted to 7.4.) Following this, cells were lysed by adding 250 μl of 0.01 M NaOH containing 0.1% Triton X-100. Aliquots were assayed for radioactivity by liquid scintillation counting (Packard Pico-Aqua, Canberra Packard AG, Zürich, Switzerland) with protein quantification. Samples were measured using a Tri-Carb2900TR Liquid Scintillation Analyzer (PerkinElmer, Inc., Boston, USA). Incubations in the presence of ouabain (1 mM), an inhibitor of Na^+/K^+ -ATPase were performed to determine Na^+/K^+ -ATPase-independent uptake of $^{86}\text{Rb}^+$. The fraction that was not inhibited by ouabain (NS) was subtracted from total counts to give the Na^+/K^+ -ATPase-mediated uptake of $^{86}\text{Rb}^+$.

1.2.7 Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All comparisons were conducted by student t-tests, and ANOVA with Tukey or Dunnett multiple comparisons test where indicated, and a value of $p < 0.05$ was considered statistically significant. Values are expressed as the mean $\pm$ SEM and are from replicate observations (typically 3-6) from independent experiments.

1.3 Results

1.3.1 Rottlerin induces astrocyte stellation

Immunocytochemistry in concert with confocal microscopy was used to determine changes in cellular morphology and EAAT expression and localization following treatment with rottlerin. In untreated cells, GFAP labelled both the cell body and major processes of the cell, with cell bodies appearing round and uniform in shape (Figure 1A). The pattern of localization seen with F-actin immunoreactivity revealed a sponge-like distribution, with strong fluorescence displaying a ring around the membrane of the cell body, together with a thick mesh of stress fibres extending across the diameter of the cell (Figure 1B). Strong EAAT1 immunoreactivity was localized to the membrane of the cell body, while weaker EAAT1 labelling was present on processes that were immunoreactive for GFAP (Figure 1A and 1B). EAAT2 had a punctate appearance, which was observed throughout the cell, being less restricted to the cell body (Figure 1A and 1B).

Treatment of astrocytes with rottlerin (100 μ M) exerted a time-dependent effect on astrocytic morphology. Rottlerin (1 and 6 h) caused an apparent increase in GFAP expression, with cells displaying a more stellate morphology when compared to untreated cells (Figure 1A). After 24 h treatment with rottlerin, GFAP-immunoreactive processes were sporadically distributed. Quantitative image analysis was performed and revealed a significant increase in GFAP intensity at 1 h (Figure 1C). After rottlerin treatment rhodamine-phalloidin staining was more significantly associated with the cell body showing redistribution of F-actin and disappearance of stress fibres (Figure 1B). Image analysis identified an increase in F-actin intensity at 6 h (Figure 1C). EAAT1 and EAAT2 both appeared to become re-organized with the changes in cytoskeletal arrangement as cells adopted a more stellate morphology. EAAT1 immunoreactivity was more visible around GFAP-positive cell bodies and processes (Figure

1A). Increased co-localization between EAAT2 with GFAP was apparent (Figure 1A). Rottlerin did not induce overlap between EAAT1 and F-actin (Figure 1B) but rottlerin induced some co-localization between EAAT2 and F-actin (Figure 1B). Reductions in GFAP and F-actin observed at 24 h are likely to be due to a minor component cell death as a reduced number of cells were observed at this time point. These data suggested that rottlerin induces cytoskeletal rearrangement including subsequent changes in EAAT distribution.

1.3.2 Rottlerin reduces EAAT activity and alters EAAT kinetics in a reversible manner

Exposure of cultured astrocytes to rottlerin (100 μ M) for 0 – 24 h produced time-dependent decreases in EAAT activity as measured by the transport of [3 H]-D-Asp with significant decreases found as early as 0.5 h ($64 \pm 10\%$ of control, $P < 0.01$) (Figure 2A). The decrease in uptake appeared to plateau between 1 and 4 h, although a further decrease in activity was observed at 24 h ($16 \pm 2.2\%$ of control, $P < 0.001$). The $t_{1/2}$ for inhibition of Glu transport was determined to be ~50 min (Figure 2A). This reduction in activity was not due to changes in cell viability (Figure 2B). Alterations in EAAT activity could occur through changes in the affinity (K_m) of the transporter and/or changes in the rate of transport (V_{max}), so kinetic analysis was performed (Figure 2C). Treatment with rottlerin caused a significant decrease in the V_{max} (control 55 ± 36 , 1 h 10 ± 3.3 , 24h 5 ± 3.7 pmol/mg protein/min $P < 0.05$), with no significant effect on the K_m of the transporter (control 44 μ M (13-150; 95% confidence intervals), 1 h 20 μ M (10-39), 24h 9.6 μ M (1.8-52)). The decrease in V_{max} was very similar at both 1 and 24 h. The decrease in V_{max} represents a decrease in the rate of transport that could arise from a pool of inactive transporters at the cell surface or from a decrease in the number of functional transporters present at the cell surface consequent to EAAT internalization (Susarla and Robinson, 2003, Gonzalez et al., 2005).

We next investigated the rate of recovery of Glu uptake following removal of rottlerin: astrocytes were treated with rottlerin (100 μ M, 6 h) after which the drug was removed (0 - 20 h) by washing cells three times with astrocytic medium. EAAT activity was analyzed over the next 20 h. Cells treated with rottlerin showed a significant decrease in [3 H]-D-Asp uptake at 6 h ($26 \pm 7.7\%$ of control, $P < 0.0001$) (Figure 2D). Once rottlerin was removed, [3 H]-D-Asp uptake returned to control levels with a rapid early increase in activity seen at 0.25 h post treatment (to $54 \pm 11\%$ of control), followed by a slower return to levels similar to control by 2 h following removal of rottlerin ($71 \pm 7\%$ of control, $t_{1/2} = 174$ min). There was no change in cell viability following rottlerin treatment or during the post-treatment stage (results not shown). Thus these findings indicated that rottlerin reversibly inhibited EAAT activity via changes in the rate of Glu transport and not via changes in affinity of the transporters.

1.3.3 Alterations in cell surface EAAT expression after rottlerin treatment do not parallel EAAT activity

The biotinylation of cell surface proteins was used together with Western blotting to examine the effect of rottlerin treatment and withdrawal on expression of EAAT1 (Figure 3 A-F) and EAAT2 (Figure 3 G-L). Western blots of biotinylated samples from 3 independent experiments identified immunoreactivity in all 3 protein fractions: total (Figure 3 A, G), intracellular (Figure 3 C, B) and cell surface (Figure 3 E, K) protein for control astrocytes, cells treated with rottlerin (6h, 100 μ M) and cells treated with and removed from rottlerin (6 h 100 μ M, 6 h recovery). Both EAAT1 and EAAT2 immunoreactivity appeared as 3 bands, representing monomers and higher molecular weight complexes. For EAAT1, all species were detected in all three fractions (Figure 3 A, C and E) where as for EAAT2 only the monomer was found in intracellular fraction and was absent from the cell surface fraction (Figure 3 G, I and K).

Significant changes in levels of total, membrane or intracellular EAAT1 expression were not observed with rottlerin treatment (6 h) or following rottlerin removal (6 h, Figure 3 A-F). Levels of total EAAT2 protein increased by approximately 2.7 fold in recovery cells compared to untreated cells (Figure 3H, $P < 0.01$). There was no statistical difference in intracellular EAAT2 levels compared to control (Figure 3J). Paradoxically, despite decreased EAAT activity at this timepoint, there was a 2.6 fold increase in EAAT2 expression at the cell surface of rottlerin-treated cells (Figure 3L, $P < 0.05$). In rottlerin recovery cells, EAAT2 protein levels at the cell surface remained increased (2.8 fold) compared to untreated cells (Figure 3L, $P < 0.05$). Overall our evidence of elevated EAAT expression at the cell surface when Glu transport was decreased is indicative of the recruitment of alternate mechanisms.

1.3.4 Interference with trafficking pathways fails to block recovery of EAAT activity

To further investigate the possible involvement of trafficking and internalisation pathways in the recycling of EAATs to the cell surface, a number of commonly used inhibitors were employed to target specific stages of the anterograde pathway (Servitja et al., 1999, Kittler et al., 2000, Casale et al., 2005). Cells were treated with rottlerin alone or in the presence of inhibitors to determine if the inhibitor could alter the recovery of EAAT activity observed following rottlerin removal. Brefeldin A (50 μ M, 24 h, Figure 4A) and nocadazole (10 μ M, 1 h Figure 4B), which block ER to Golgi and Golgi trafficking, respectively, had no effect on the recovery of EAAT activity following rottlerin removal. Monensin (100 μ M, 2 h, Figure 4C), which amongst other actions, interferes with the *trans*-Golgi region and endosomal trafficking (Casale et al., 2005), increased the rate of recovery of EAAT activity ($t_{1/2} = 13.38$ mins, Figure 4C, $P < 0.01$) with significant effects seen at 0.25, 1 and 2 h. Changes in cell viability were not observed with any treatment (data not shown).

In related experiments, astrocytes were treated with hypertonic concentrations of sucrose (0.45 M, 0.5 h) to determine if the reduction in EAAT activity observed with rottlerin treatment could be blocked by inhibiting endocytosis. Treatment with rottlerin caused a significant decrease in [^3H]-D-Asp uptake (~35%, $P < 0.01$), while sucrose treatment had no effect (Figure 4D). Sucrose was unable to block the inhibitory effects of rottlerin when added together with rottlerin (Figure 4D, ~35%, $P < 0.01$). Although however minor reductions in cell viability were observed with both sucrose (~10%, $P < 0.01$) and rottlerin + sucrose (~15%, $P < 0.001$) treatments (Figure 4E), the effects on EAAT activity were not associated with changes in cell viability. These data indicated that rottlerin-induced changes in EAAT activity were not due to intracellular trafficking of transporters away from the cell surface and further suggested another mechanism of inhibition.

1.3.5 Rottlerin inhibits the Na^+/K^+ -ATPase which recovers following rottlerin removal

The ability of rottlerin to act as a metabolic uncoupler, together with the evidence that monensin, a Na^+ ionophore (Pressman and Fahim, 1982), improves the recovery of EAAT activity following rottlerin treatment led us to consider the novel hypothesis that Na^+/K^+ -ATPase regulation underlies the altered EAAT activity. Therefore the effect of rottlerin and monensin on the activity of the Na^+/K^+ -ATPase was investigated.

$^{86}\text{Rb}^+$ uptake into astrocytes was measured as an index of the activity of the Na^+/K^+ -ATPase in untreated cells, cells treated with rottlerin (100 μM , 6 h) or monensin (100 μM , 2h), or cells treated with both rottlerin (100 μM , 6 h) + monensin (100 μM , 2h) (Figure 5A). Rottlerin treatment significantly reduced $^{86}\text{Rb}^+$ uptake by ~55% ($P < 0.001$), whereas treatment with monensin increased $^{86}\text{Rb}^+$ uptake by ~24% ($P < 0.01$). Rottlerin + monensin treatment

decreased $^{86}\text{Rb}^+$ uptake into cells by ~82% when compared to untreated cells ($P<0.001$). A MTT assay was performed to determine the effect of treatment on cell viability and revealed no significant effect of any treatment on the viability of the cells (Figure 5B).

Primary astrocyte cultures were treated with rottlerin (100 μM , 6 h) or rottlerin + monensin (100 μM , 2 h) and $^{86}\text{Rb}^+$ uptake was determined (Figure 5C). Cells treated with rottlerin alone showed a significant decrease in $^{86}\text{Rb}^+$ uptake at the treated time-point (zero recovery) of ~50% ($P<0.05$). Following rottlerin removal, Na^+/K^+ -ATPase activity rapidly returned to control levels within 15 min. Na^+/K^+ -ATPase activity continued to increase beyond control levels, reaching ~50% above control levels 4 and 6 h after rottlerin removal. In cells treated with rottlerin + monensin, a significant decrease in $^{86}\text{Rb}^+$ uptake of ~50% was observed at the treated time-point ($P<0.001$). Following rottlerin removal, when cells were exposed to monensin alone, no increase in Na^+/K^+ -ATPase activity was observed: $^{86}\text{Rb}^+$ uptake remained at ~50% of control cells 6 h after rottlerin removal. MTT assays revealed small, non-significant decreases in cell viability in both treatment groups (Figure 5 D, $P>0.05$) suggesting that treatments had no effect on cell viability over the 6 h recovery period. A small but lost-lasting decrease (10%) in total ATP levels was seen in both rottlerin treated and recovery cells (Figure 5E): an effect which matched the time-course of recovery of Glu uptake and elevation of cell surface expression of EAAT2. In summary, rottlerin reversibly inhibited the activity of the Na^+/K^+ -ATPase suggestive of a link between the loss of Glu transport and the Na^+/K^+ -ATPase.

1.4 Discussion

1.4.1 Loss of Glu transport linked to cytoskeletal changes and Na^+/K^+ -ATPase inhibition

Under pathological conditions astrocytic EAATs display decreased activity together with reactive astrogliosis (Beart & O'Shea, 2007) and in experimental models genetic or pharmacological down-regulation of EAAT activity results in altered Glu metabolism and astrocytic function with neuronal damage (Danbolt, 2001; Beart & O'Shea, 2007; Sheldon & Robinson, 2007). Previous work from our group has greatly advanced the case for a link between changes in astrocytic morphology and EAAT activity (O'Shea et al., 2006, Lau et al., 2010, Lau et al., 2011) supporting earlier evidence (Schlag et al., 1998, Hughes et al., 2004, Zhou and Sutherland, 2004). We demonstrate for the first time that rottlerin causes changes in astrocytic morphology typical of development of a reactive phenotype in astrocytes. Changes in astrocytic morphology consistent with stellation were found, including elongation of cells, increased GFAP immunoreactivity, loss of F-actin stress fibres and condensation of labelling around the cell body (Tsai et al., 2006). Moreover, we showed that rottlerin caused a rapid decrease in EAAT activity, an affect that was fully reversible following rottlerin withdrawal and not related to cellular toxicity, but due to an altered cytoskeleton and inhibition of Na^+/K^+ ATPase activity.

Alterations in transporter activity are commonly associated with changes in the expression and/or localization of the transporter protein in particular, loss of transporter function is often associated with a reduction in the cell surface expression of transporter protein (Sotnikova et al., 2006). Indeed, recent time-lapse evidence shows accumulation of EAAT2 clusters in astrocytic processes during neuronal activation and subsequent reduction in expression and changes in localization during inactivation states (Benediktsson et al., 2012). We, like others (Martinez-Villarreal et al., 2012), have observed such vesicular-like clusters for EAAT2,

including after rottlerin treatment (data not shown). For EAATs many of these issues remain to be resolved and while there is evidence for EAAT molecular complexes (Bauer *et al.*, 2012; Genda *et al.*, 2011; Rose *et al.*, 2009), there is a relative scarcity of information on the regulation of their cell surface expression and trafficking (Beart & O'Shea, 2007; Sheldon & Robinson, 2007).

1.4.2 Homeostatic increases in EAAT2 expression with Glu transport inhibition

Previously, under conditions where Glu uptake was compromised we found evidence for a homeostatic mechanism maintaining EAAT expression in the presence of altered astrocytic morphology (Lau *et al.*, 2010; Zagami *et al.*, 2009). In primary cultures of rat astrocytes, rottlerin had inhibitory effects on EAAT1 activity, decreased EAAT1 cell surface expression and increased EAAT degradation (Susarla and Robinson, 2003). Our primary astrocytic cultures of astrocytes were more mature and expressed both EAAT1 and EAAT2, allowing us to explore the effects of rottlerin on both transporters. Data here support previous studies showing the inhibitory effect of rottlerin on the activity of the astrocytic transporters (~50% after 50 min, 100 μ M) and also the reduction in the rate of transport. However, biotinylation and western blotting revealed that rottlerin treatment was not associated with EAAT2 or EAAT1 decreases in the cell surface fraction. Paradoxically, rottlerin caused a large increase in cell surface EAAT2. This result was surprising as elevated levels of total EAAT protein or an increase in EAAT protein at the cell surface activity are often associated with an upregulation in EAAT activity (Davis *et al.*, 1998, Robinson, 2002). Removal of rottlerin restored EAAT activity, but was accompanied by maintenance rather than a further elevation of EAAT expression at the cell surface. Thus the inhibition caused by rottlerin is not due to a loss of transporter expression at the cell surface but rather a loss of function. These findings were further supported by evidence that drugs targeting the endoplasmic reticulum, Golgi

apparatus and endosomes (brefeldin, nocadazole, sucrose), intracellular compartments classically linked to trafficking and internalization, did not alter rottlerin-induced inhibition of Glu uptake or recovery. Indeed, Susarla and Robinson (2003) reported that rottlerin had no effect on the transferrin receptor and concluded that its action on EAAT1 was not mediated via classical Rab recycling mechanisms. Our data suggest that, under the conditions employed, the inhibition of EAAT activity by rottlerin is not due to EAAT trafficking.

During our analysis of possible mechanisms linked to protein trafficking and internalization, we observed that monensin significantly enhanced recovery of EAAT activity following rottlerin withdrawal. Although monensin was employed as an inhibitor of trafficking from the Golgi (Mollenhauer et al., 1990), it also has a role as a Na^+ ionophore (Pressman, 1976). The ability of monensin to regulate Na^+ and other monovalent cations might underlie its ability to increase the rate of EAAT recovery following rottlerin removal. EAAT activity is exquisitely dependent on ionic gradients and the Na^+/K^+ -ATPase (Abe and Saito, 2000, Danbolt, 2001). Indeed EAATs are directly coupled to Na^+/K^+ -ATPase and direct inhibition of Na^+/K^+ -ATPase leads to a reduction in Glu uptake and transporter reversal (Gemba et al., 1994, Li et al., 1999, Rossi et al., 2000) as well as an increase in vulnerability of neurons to Glu toxicity (Novelli et al., 1988, Kohmura et al., 1990, Brines et al., 1995). Therefore we investigated whether rottlerin affected the Na^+/K^+ -ATPase since inhibition of the Na^+/K^+ -ATPase could inactivate EAATs without reducing EAAT expression at the cell surface.

1.4.3 Na^+/K^+ -ATPase activity and ATP are important for Glu transport

Rottlerin, often considered a PKC δ inhibitor, is recognised to have numerous pharmacological actions (Bain et al., 2007, Soltoff, 2007). Its actions include an ability to uncouple mitochondrial respiration from oxidative phosphorylation by lowering the H^+

gradient, increasing O₂ consumption and reversing the action of ATP synthase, resulting in a loss of ATP (Soltoff, 2001) and depolarization of the mitochondrial membrane potential (Liao et al., 2005). We show that treatment of astrocytes with rottlerin inhibited Na⁺/K⁺-ATPase activity and caused a small but sustained reduction in total ATP. The reduction in activity is consistent with the mitochondrial uncoupling effect of rottlerin, resulting in a decrease in ATP to drive the Na⁺/K⁺-ATPase. Recent work by (Bauer et al., 2012) shows evidence of EAATs existing as a macromolecular complex with the Na⁺/K⁺-ATPase in close proximity to mitochondrial and local ATP pools. While there were only minor overall effects on MTT activity, an index of total mitochondrial respiratory chain function, and ATP, given the strong dependence of Glu transport on glycolytic ATP, a local deficit may affect EAAT function (Hertz et al., 2007, Genda et al., 2011). EAAT2 co-compartmentalizes with Na⁺/K⁺-ATPase, glycolytic enzymes and mitochondria, providing a mechanism to spatially match energy and ionic dependencies to transport demands (Genda et al., 2011). The initial loss in ATPase activity coincides with the loss in EAAT activity and suggests that these observations are mechanistically linked. Rottlerin has previously been shown to inhibit Na⁺/K⁺-ATPase activity and Glu uptake (Nguyen et al., 2009). Nguyen et al. (2009) linked the loss of Glu uptake with rottlerin treatment to a reduction in EAAT1 cell surface expression, however as discussed above, there was no evidence here for such an effect using careful biochemical analyses which showed a key role for EAAT2.

1.4.4 Monensin suggests role for Na⁺ in EAAT inhibition

We show for the first time that removal of rottlerin rapidly reverses the inhibition on the Na⁺/K⁺-ATPase, which reaches control levels within 15 min, and becomes further elevated above that of untreated cells, with the stimulation of Na⁺/K⁺-ATPase maintained even 6 h after removal of rottlerin suggestive of uncoupling from transporter activity. Since Na⁺

movements in microdomains close to the plasma membrane may reflect activation of Na^+/K^+ -ATPase (Rose and Karus, 2013), here once Na^+ rises it may stimulate local astrocytic glycolysis to fuel reinstatement of EAAT activity. While monensin increased the uptake of $^{86}\text{Rb}^+$, consistent with its ability to rapidly stimulated Na^+/K^+ -ATPase (Pressman, 1976, Brodie et al., 1987), it was unable stimulate the Na^+/K^+ -ATPase when cells were treated together with rottlerin or following rottlerin removal. Thus the ability of monensin to increase the rate of recovery of EAAT activity following rottlerin treatment is not via stimulation of the Na^+/K^+ -ATPase and but rather another mechanism, perhaps its activity as a Na^+/H^+ exchanger (Mollenhauer et al., 1990).

1.4.5 Conclusions

This study provides significant new evidence of the relationship between Na^+ , the Na^+/K^+ -ATPase and Glu transport supporting the concept of a tight inter-dependent coupling between these important systems. The uncoupling of these two key processes is likely to lead to a loss of Glu uptake and excitotoxicity, and changes in the interaction between EAATs and the Na^+/K^+ -ATPase during pathologies an important target for further investigation. Overall, based upon our findings it is possible that there may be three temporal components of the astrocytic response: (1) cytoskeletal reorganization, (2) increased EAAT2 expression and (3) elevated Na^+/K^+ -ATPase activity.

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Conflict of interest

The authors state no conflict of interest.

Figures and Figure legends

Fig 1. (A) Effects of rottlerin treatment on the morphology of mouse astrocytes. GFAP (red A), F-actin (red B) and EAAT1 or EAAT2 (green) were labelled in control and rottlerin-treated cells (treatment duration shown above images). White arrows indicate appearance of processes (A), or loss of stress fibres (B), * indicate co-localization between GFAP or F-actin and EAAT1/2. Mag = 40x, scale bar = 30 μ m. (C and D) Image analysis of the effects of rottlerin treatment on the intensity of cytochemical labelling. Effects of rottlerin on the intensity of GFAP and F-actin (C), and EAAT1 and EAAT2 (D) labelling in mouse astrocytes. Data shown are mean intensities of triplicate determinations from at least three individual experiments presented as percentage control $\pm$ SEM. One-way ANOVA revealed a significant effect of treatment on GFAP ($P < 0.01$), F-actin ($P < 0.05$), EAAT1 ($P < 0.05$) and EAAT2 ($P < 0.0001$). Asterisks (*) indicate significantly different from control, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), Dunnett's Multiple Comparison post-hoc test.

Fig 2. Time-dependent effects of rottlerin and rottlerin removal on specific [3 H]-D-Asp uptake and cell viability in cultured astrocytes. Effect of rottlerin (100 μ M) over time (0.5 - 24 h) on the specific uptake of [3 H]-D-Asp (A), cell viability (B) and kinetics (C). (A+B) Data shown are means of triplicate determinations of four individual experiments presented as percentage control $\pm$ SEM. One-way ANOVA revealed a significant effect of rottlerin treatment ($P < 0.0001$) on [3 H]-D-Asp uptake. Asterisks (*) indicate significantly different from control, Dunnett's Multiple Comparison post-hoc test. (C) Data were fitted to a homologous competitive binding curve for one class of binding sites constrained for the determined values of [3 H]-D-Asp (50 nM) using GraphPad Prism v 5.0, and which determined the V_{max} and K_m of transport under each condition. Data are expressed as mean $\pm$ SEM from 4 independent experiments. (ROTT – rottlerin treatment). (D) Effect of the

removal of rottlerin on the specific uptake of [3 H]-D-Asp. Data shown are means of triplicate determinations from four individual experiments presented as percentage control $\pm$ SEM. The checkered bar represents cells treated with rottlerin (6h) with no recovery. The black bars represent the recovery phase of the treatment. (A) Two-way ANOVA indicated the different time points were significantly different ($P < 0.05$). Asterisks (*) indicate significantly different from control, Dunnett's Multiple Comparison test, (***) $P < 0.001$.

Fig 3. Effect of rottlerin on EAAT1 (A-F) and EAAT2 (G-L) expression. Astrocytes were treated with rottlerin (100 μ M, 6h) or allowed to recovery following rottlerin removal (6h) and biotinylation of cell surface proteins was performed to separate cell fractions. Western blots show immunoreactivity for EAAT1/2 in whole cell (A and G), intracellular (C and I) and cell surface (E and K) fractions from homogenates of cultured astrocytes under control and rottlerin-treated conditions. Samples are from 3 independent experiments ($n = 6$ wells/condition) and were loaded independently for each condition allowing comparison within and between experiments. Histograms show densitometric measurements for all EAAT1/2 monomeric, dimeric and trimeric proteins. Data are presented as percentage control of mean $\pm$ SEM. Paired t-test revealed a significant effect of rottlerin treatment on total ($P < 0.05$) and cell surface ($P < 0.05$) expression of EAAT2. Asterisks (*) indicate significant difference from control, ** $P < 0.05$.

Fig 4. Effects of rottlerin and inhibitors of intracellular trafficking on [3 H]-D-Asp uptake and astrocytic viability. Astrocytes were treated with rottlerin $\pm$ (A) brefeldin A (BFA, 50 μ M, 24h), (B) nocadazole (NDZ, 10 μ M, 1h) or (C) monensin (MON, 100 μ M, 2h) and recovery of [3 H]-D-Asp uptake determined. (D) In similar experiments, astrocytes were treated with rottlerin (100 μ M, 0.5h, black bars), sucrose (0.4M, 0.5h, grey bars) or their combination

(checkered bars) and specific uptake of [^3H]-D-Asp (D) and cell viability (E) determined. Data shown are means of triplicate determinations of four individual experiments presented as percentage control. (C) Two-way repeated measures ANOVA revealed a significant difference between rottlerin and rottlerin + monensin treatment groups ($P < 0.001$), Tukey multiple comparison test. Asterisks (*) represent significant differences to rottlerin treatment (* $P < 0.05$, ** $P < 0.01$). (D and E) One-way ANOVA revealed a significant effect of rottlerin ($P < 0.01$) and rottlerin + sucrose (checkered bars, $P < 0.001$) treatment on [^3H]-D-Asp uptake, and a significant effect of sucrose ($P < 0.01$) and rottlerin + sucrose ($P < 0.001$) treatment on cell viability, Dunnett's Multiple Comparison post-hoc test. (* $P < 0.05$).

Fig 5. Effects of rottlerin and monensin treatment on Na^+/K^+ -ATPase activity and astrocytic viability. Astrocytes were treated with rottlerin (100 μM , 6h), monensin (100 μM , 2h) or their combination and $^{86}\text{Rb}^+$ uptake and MTT activity, indices of Na^+/K^+ -ATPase (A) and viability (B), respectively, determined. Recovery of both activities was studied up to 6 h (C) and (D). Data are means of triplicate determinations in each of three independent experiments presented as percentage control $\pm$ SEM. (A and B) One-way ANOVA of original data revealed a significant effect of rottlerin treatment ($P < 0.001$), monensin ($P < 0.01$) and rottlerin + monensin treatment ($P < 0.001$) on $^{86}\text{Rb}^+$ uptake, but no effect of any treatment on cell viability ($P > 0.05$) compared to control. Significant differences to control are represented as (*), to rottlerin are represented as (#) and to monensin represented as (^), Tukey multiple comparisons test. (C and D) Two-way ANOVA of original data revealed a significant difference between rottlerin and rottlerin + monensin treatment on $^{86}\text{Rb}^+$ uptake, but no effect of any treatment on cell viability ($P > 0.05$), Bonferroni post-test, (***) $P < 0.001$.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Highlights from paper - **Links between L-glutamate transporters, Na⁺/K⁺-ATPase and cytoskeleton in astrocytes: evidence following inhibition with rottlerin.** (Sheean et al)

- Rottlerin induces cytoskeletal changes consistent with astrocyte activation and stellation.
- Rottlerin inhibits glutamate uptake via a decreased rate of transport, not a loss in cell surface glutamate transporters.
- Loss of glutamate uptake was reversible and enhanced by co-treatment with monensin.
- Na⁺/K⁺-ATPase activity and ATP levels are also reduced with exposure to rottlerin.
- Recovery of Na⁺/K⁺-ATPase activity preceded Glu uptake rescue supporting partial uncoupling of these processes.

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Title:

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