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Increased excitatory amino acid transport into murine prion protein knockout astrocytes cultured in vitro

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Abstract

Prion protein (PrP) is expressed on a wide variety of cells and plays an important role in the pathogenesis of transmissible spongiform encephalopathies. However, its normal function remains unclear. Mice that do not express PrP exhibit deficits in spatial memory and abnormalities in excitatory neurotransmission suggestive that PrP may function in the glutamatergic synapse. Here we show that transport of D-aspartate, a non-metabolized L-glutamate analog, through excitatory amino acid transporters (EAATs) was faster in astrocytes from PrP knockout (PrPKO) mice than in astrocytes from C57BL/10SnJ wildtype (WT) mice. Experiments using EAAT subtype-specific inhibitors demonstrated that in both WT and PrPKO astrocytes, the majority of transport was mediated by EAAT1. Furthermore, PrPKO astrocytes were more effective than WT astrocytes at alleviating L-glutamate-mediated excitotoxic damage in both WT and PrPKO neuronal cultures. Thus, in this in vitro model, PrPKO astrocytes exerted a functional influence on neuronal survival and may therefore influence regulation of glutamatergic neurotransmission *in vivo*.

Keywords

Prion protein; excitatory amino acid transporters; astrocyte; excitotoxicity; NMDA receptor

Introduction

Prion protein (PrP) is an evolutionarily conserved, glycoposphatidylinositol-anchored membrane protein expressed in the mammalian nervous system, as well as in many other tissues. Expression in the CNS begins early in development and remains throughout adulthood (Miele et al. 2003). Despite its wide expression, the function of PrP remains enigmatic. While PrP knockout mice (PrPKO) do not suffer from overt disturbances in development or behavior (Bueler et al. 1992; Manson et al. 1994), they do exhibit subtle abnormalities which suggest PrP involvement in various cellular processes including neuroprotection against oxidative stress, support for neurite outgrowth, and maintenance of myelinated axons (reviewed in Aguzzi et al. 2008). In particular, PrPKO mice suffer from deficits in hippocampal spatial memory (Criado et al. 2005) and NMDA receptor-related neurophysiological and behavioral abnormalities, suggestive of a function of PrP within the

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glutamatergic synapse (Carleton et al. 2001; Collinge et al. 1994; Criado et al. 2005; Khosravani et al. 2008; Mallucci et al. 2002; Manson et al. 1994). These dysfunctions in PrPKO mice could be attributable to a variety of underlying mechanisms involving aberrant signaling through glutamate receptors on neurons and/or abnormal neurotransmitter regulation by glutamate transporters on astrocytes.

One of the major functions of astrocytes is to sequester the excitatory neurotransmitter L-glutamate intracellularly and thereby regulate activation of excitatory amino acid receptors (Anderson and Swanson 2000; Bridges and Esslinger 2005; Eulenburg and Gomeza 2010). Clearance of L-glutamate is primarily mediated by high affinity, sodium-dependent excitatory amino acid transporters (EAATs). Two of the five identified transporter isoforms, EAAT1 and EAAT2, are highly expressed on astrocytes in the cerebellum, hippocampus and cerebral cortex. Both transporters are located on glial plasma membranes and, in combination, are responsible for the bulk of L-glutamate transport in the CNS (Danbolt 2001). The EAATs play a pivotal role in controlling excitatory signaling as highlighted both by their anatomical specificity (Anderson and Swanson 2000) and ability to protect neurons from glutamate-mediated excitotoxicity (Robinson et al. 1993; Rothstein et al. 1996).

An inability of astrocytes to regulate L-glutamate neurotransmission might lead to deficits in excitatory neurotransmission and, in particular may contribute to deficits observed in PrPKO mice. In the present experiments, we carried out detailed kinetic studies on EAAT-mediated glutamate transport in astrocytes prepared from C57BL/10SnJ wildtype (WT) mice and from PrPKO mice containing 98.5% sequence identity to the C57BL/10SnJ genotype. We found that astrocytes from PrPKO mice exhibited higher rates of sodium dependent transport of the EAAT-selective substrate D-aspartate than did astrocytes from WT PrP expressing mice. Using inhibitors selective for EAAT subtypes, we examined the type of transporters functioning in both PrPKO and WT astrocytes and investigated whether PrP related changes in activity could influence neuronal vulnerability to glutamate-mediated excitotoxicity. These results provide a potential explanation for the behavioral abnormalities observed in PrPKO mice.

Materials and Methods

Animals

C57BL/10SnJ mice (WT) were purchased from Jackson Labs (Bar Harbor, ME). Homozygous PrP null (PrPKO) mice on the 129/Ola background (Manson et al. 1994) were backcrossed nine times to C57BL/10SnJ mice selecting for the PrPKO allele by PCR identification at each cross (Race et al. 2009). Single nucleotide polymorphism (SNP) analysis was performed on DNA from PrPKO mice, and results were compared to DNA from C57BL/10SnJ mice (Taconic Farms Inc., Rensselaer, NY). Non-C57BL/10SnJ SNPs, i.e. from the 129/Ola mouse strain donor of the knockout Prnp gene, were identified only on chromosome 2, adjacent to the Prnp gene locus.

Astrocyte and Neuron Cell Cultures

Mixed glial cells were harvested from the cortices of WT and KO 1–2 day old mice using modifications of the method of McCarthy and de Vellis (McCarthy and de Vellis 1980). Cortices, with meninges removed, were triturated, plated in T-25 flasks with DMEM/F12 (Invitrogen, Carlsbad, CA) containing 15% fetal bovine serum (HyClone, Omaha, NE) and maintained in a 5% CO₂ incubator at 37°C. Approximately seven days later, when cultures were confluent, microglia and oligodendrocytes were removed from the astrocyte cultures by orbital shaking (overnight, 250 rpm). Remaining astrocytes were harvested with trypsin and reseeded at 1×10^5 cells/well in 12 well plates with fresh media changed every three

days. In some experiments, upon reaching confluence approximately seven days post-seeding, primary astrocytes were analyzed for EAAT expression and D-aspartate uptake. In other experiments, confluent primary astrocyte cultures were treated with 0.25mM dibutyryl cyclic AMP (dbcAMP) (Sigma Aldrich, St. Louis, MO) for an additional ten days with media changes including dbcAMP every three days. In other experiments, media from confluent astrocytes was completely replaced with neuron-conditioned media (NCM) and cultured for an additional five days to increase EAAT2 expression (Gegelashvili, et al., 1997; Yang, et al., 2009). See below for preparation of NCM.

Primary cortical neurons were prepared as described previously (Kaech and Banker 2006). Briefly, whole cerebral neocortices removed from either WT or PrPKO embryos (14–16 days gestation) were digested for 30 minutes with 0.25% trypsin (Invitrogen, Carlsbad, CA) and washed four times in Hanks Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA). For excitotoxicity experiments, cortical cells, 5×10^4 cells/well, were plated onto a bed of 2.5×10^4 purified astrocytes that had been plated in 24 well plates two days earlier. For transporter inhibition, mRNA, and protein experiments, 1×10^5 cortical cells/well were plated in 12 well plates seeded two days earlier with 5×10^4 purified astrocytes. Neuron-containing cortical cells were initially seeded in MEM with 10% heat-inactivated horse serum (HyClone, Omaha, NE) and 0.25% glucose. The media was completely replaced 6 hours later with Neurobasal media (500 μ l) (Invitrogen, Carlsbad, CA) supplemented with Glutamax (Invitrogen, Carlsbad, CA), B-27 with antioxidants (Invitrogen, Carlsbad, CA) and 5 μ M cytosine arabinoside (Sigma Aldrich, St. Louis, MO) to halt cell proliferation. Cells were fed once at day 7 by replacing half the media with fresh media. For glutamate excitotoxicity experiments as well as for D-aspartate transport inhibition experiments mixed astrocyte-neuronal cultures were used 14 days later, coinciding with NMDA receptor expression (Choi et al. 1987). In some experiments, neuron-conditioned media (NCM) from 14-day C57BL/10 or PrPKO neuron-astrocyte co-cultures was harvested, cleared by centrifugation at 1200rpm and placed onto confluent astrocytes of the same genotype.

PrP immunocytochemistry

PrP staining was performed on live primary astrocytes using a 1:1000 dilution of humanized monoclonal anti-PrP antibody D13 (Williamson et al. 1998). Following 1 hour incubation at room temperature, cells were fixed in 3.7% formaldehyde in PBS for 20 minutes, washed in PBS, permeabilized in 0.1% Triton x100, 0.1% sodium citrate for ten minutes, washed in PBS and labeled with rabbit anti-GFAP (1:1500) (Dako, Carpinteria, CA) followed by Alexa Fluor568 conjugated goat anti-rabbit IgG (1:3000) (Invitrogen, Carlsbad, CA) to identify astrocytes and Alexa Fluor488 goat anti-human IgG (1:3000) (Invitrogen, Carlsbad, CA) to visualize PrP staining. Fluorescent images were photographed on an upright microscope (Olympus BX51) with a 10X objective using Microsuite Analysis software. Control wells stained with secondary antibodies alone did not show immunofluorescence.

Surface PrP staining and FACS analysis

Purified WT and KO astrocytes were rinsed with PBS and removed from T-25 flasks by incubation with 5mM EDTA for 15 minutes at 37°C. PrP staining was performed on 200,000 live primary astrocytes using 1 μ g antibody D13 in 50 μ L PBS. Following 1 hour incubation at 37°C, cells were washed in PBS, fixed in 3.7% formaldehyde in PBS for 20 minutes, blocked in 0.1M glycine in PBS for 30 minutes and incubated for 1 hour at RT with 1 μ g Alexa Fluor488 goat anti-human IgG (Invitrogen, Carlsbad, CA) in 50 μ L PBS. Control tubes of WT and KO astrocytes were fixed, blocked and incubated with Alexa Fluor488 goat anti-human IgG alone. After washing again, cells were analyzed using a FACSCanto II flow cytometer (Becton Dickinson, San Jose CA) and data was analyzed using FlowJo (Tree Star, Ashland, OR).

Na⁺ dependent D-aspartate uptake assays

Confluent astrocytes in 12 well plates were rinsed with a physiological transport buffer (138 mM NaCl, 11 mM D-glucose, 5.3 mM KCl, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 1.1 mM CaCl₂, 0.7 mM MgSO₄, 10 mM HEPES, pH 7.4) and pre-incubated at 37 °C for 5 min. Transport rates were determined using ³H-D-aspartate, which is effectively transported as an EAAT substrate, yet not metabolized by cells following uptake (Koch et al. 1999). To initiate transport, preincubation buffer was replaced with buffer containing ³H-D-aspartate (5–300 μM, 2–12 μCi/ml). Following a 5 minute incubation at 37°C, uptake was terminated by 3 consecutive washes with ice-cold buffer. Cells were lysed with 0.4N NaOH for 24 hours and analyzed for radioactivity by liquid scintillation counting and for protein by the bicinchoninic acid method (Pierce, Rockford, IL). Uptake [pmol D-asp/min/mg protein] was calculated and corrected for background radiolabel accumulation at 4°C. Previous studies confirmed that uptake measured under these conditions was linear with respect to time and protein content (Esslinger et al. 2005). Values are reported as mean ± SEM pmol/min/mg with each “n” value equaling the number of determinations, each done in duplicate. Data was fit to the Michaelis-Menten equation using non-linear regression (Prism 5).

Transport of 5 μM D-aspartate was also measured in the presence of selective EAAT inhibitors: L-serine-O-sulfate (LSOS) (Sigma Aldrich, St. Louis, MO), dihydrokainate (DHK) (Tocris Bioscience, Ellisville, MO), and TBOA (Tocris Bioscience, Ellisville, MO), each of which was added at the indicated concentrations simultaneously with 5 μM ³H-D-aspartate.

Quantitative RT-PCR Analysis

For analysis of mRNA by RT-PCR, cultured astrocytes were harvested with trypsin and lysed using Qiashredder (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy mini kit with DNase treatment (Qiagen, Valencia, CA). RNA was reverse-transcribed into cDNA using reverse transcription reagents with random hexamers (Applied Biosystems, Foster City, CA). The cDNA product was then amplified in a new tube using gene expression assays specific for the EAAT1 gene (Slc1a3), EAAT2 gene (Slc1a2), and mouse β-actin gene (Applied Biosystems, Foster City, CA). Gene expression was quantified using the first cycle number at which each sample reached a fixed fluorescence threshold (C_T). The quantity of expression of each gene was normalized to mouse β-actin (ΔC_T). Fold expression = 2^{-ΔC_T}

Immunoblotting

To detect EAAT1 and EAAT2 proteins, cultured astrocytes were lysed in 1.0% SDS in 0.1M sodium phosphate buffer, pH 7.4, containing 1X Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL). Protein concentrations were determined using a modified Lowry protein assay with BSA as a standard. Lysates were then adjusted to a concentration of 0.03M DTT, incubated for 1 hour at room temperature, and stored at –80°C until needed. Samples were then thawed and diluted 1:1 in 2X Laemmli Buffer containing 2% SDS, 0.06 M DTT. Samples were incubated for 30 minutes at 58°C, and 10 μg of protein from each sample was separated by 10% acrylamide SDS-PAGE in 25mM Tris, 192mM glycine and 0.1% SDS. Separated proteins were transferred to nitrocellulose using a Bio-Rad Trans Blot cell (4°C, 15V, 16 hours) and blocked for 2–3 hours in 5% Dry Milk in Tris-buffered saline-0.1% Tween 20 (TTBS) before probing. Immunoblots were probed with anti-GLAST (EAAT1) antibody (1:2000, Tocris Cookson, St Louis, MO) or anti-GLT-1(EAAT2) antibody (1:2000, Tocris Cookson, St Louis, MO) in TTBS, rinsed twice with 150 ml TTBS (RT, 30 minutes each), probed with secondary antibody goat anti-rabbit Ig conjugated with IRDye 800CW (1:10,000; LI-COR Biosciences) in TTBS, and finally rinsed three times with 150 ml TTBS

(RT, 30 minutes each). Immunoreactive bands were detected and quantified using a LI-COR Odyssey Infrared Imaging System and software.

L-Glutamate-mediated excitotoxicity

After 14 days *in vitro*, astrocyte-neuron co-cultures described above were exposed to varying concentrations of L-glutamate (10–50 μ M) in 500 μ l fresh Neurobasal (Invitrogen, Carlsbad, CA) media without B27 or Glutamax for ten minutes at room temperature. Following exposure, cells were gently rinsed with HBSS, after which half their original media (250 μ l) was added back to the well combined with 250 μ l fresh Neurobasal media with B27 supplement and Glutamax to maintain original culture conditions. Twenty-four hours following L-glutamate exposure, surviving neurons were fixed with 3.7% formaldehyde for 15 minutes, washed with PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for ten minutes, rinsed with PBS, and labeled with mouse anti-MAP2 (1:1000) (Millipore, Billerica, MA) to identify neurons, rinsed with PBS, and incubated 20 minutes with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:3000) (Invitrogen, Carlsbad, CA). Nuclei were visualized by incubation with DAPI (Invitrogen, Carlsbad, CA). To count neurons, three randomly chosen fields per well were photographed. Surviving MAP2-positive neuronal cell bodies which colocalized with DAPI stained nuclei were quantified (Shin et al. 2005). Neuronal survival following L-glutamate exposure was expressed as percent relative to neuronal counts in control wells not exposed to L-glutamate. Data were obtained from 4 independent co-culture experiments.

Statistical Analysis

Statistical analysis was performed using non-parametric Mann-Whitney tests. Calculations were performed on Prism GraphPad software (version 5). Statistical significance was reported for values $P < 0.05$.

Results

PrP expression on primary astrocytes

To confirm PrP expression on WT primary C57BL/10 astrocytes *in vitro*, cortical astrocytes were purified from 1–2 day old mice and cultured *in vitro* for 7 days as described in Methods. Astrocytes from PrPKO mice were used as negative controls. The majority of cells in both WT and PrPKO cultures were GFAP-positive astrocytes, and PrP immunoreactivity was only observed on WT and not on PrPKO astrocytes (Figure 1A). Similar results were also seen when primary WT and PrPKO astrocytes were examined by flow cytometry where live cells were labeled with D13. Surface expression of PrP was only observed on WT astrocytes (Figure 1B).

Comparison of EAAT activity in WT and PrPKO astrocytes

The potential influence of PrP expression on L-glutamate homeostasis was studied by analyzing EAAT-mediated transport of D-aspartate in astrocytes prepared from WT and PrPKO mice. Transport rates between WT and PrPKO astrocytes clearly diverged at concentrations of D-aspartate greater than 50 μ M (Figure 2A). When fit to the Michaelis-Menten equation, the $V_{\max}$ values were 1.7 fold higher in the PrPKO astrocytes compared to WT astrocytes (687 vs. 407 pmol/min/mg, Table 1 and Figure 2C).

As treatment of primary astrocytes with dibutyryl-cyclic AMP (dbcAMP) produces morphological and biochemical changes more representative of astrocytes found *in vivo* (e.g. increased expression of EAATs, GFAP, glutamine synthetase, and neurotransmitter receptors) (Daginakatte et al. 2008; Hosli et al. 1997; Jackson et al. 1995; Khelil et al. 1990; Le Prince et al. 1991; Miller et al. 1994; Schlag et al. 1998; Swanson et al. 1997), WT and

PrPKO astrocytes treated for ten days with dbcAMP (0.25mM) were examined. Transport rates in the untreated cells were not significantly altered by the additional ten days in culture (data not shown). In transport experiments, PrPKO astrocytes treated with dbcAMP exhibited a 2.5 fold increase in $V_{\max}$ for D-aspartate transport when compared to WT astrocytes treated with dbcAMP (1768 vs. 697 pmol/min/mg, Figure 2B, Figure 2D, and Table 1). This increase was larger than the 1.7 fold increase observed in untreated astrocytes (Table 1). Thus, the highest $V_{\max}$ for D-aspartate transport was found in cells lacking PrP that had been treated with dbcAMP.

In contrast to the variations observed in $V_{\max}$ between WT and PrPKO astrocytes, both before and after treatment with dbcAMP, K_m values remained essentially unchanged, except for the PrPKO astrocytes that had been treated with dbcAMP (Table 1). Although this observed increase in K_m may reflect a functional change in the transporter, in this instance it could also be attributable to the increased EAAT activity. Though a uniform assay protocol was used to kinetically characterize all of the cultures, if the intracellular accumulation of substrate in those astrocytes exhibiting the highest rates of transport became significant enough, it could slow the uptake of the radiolabel and produce an apparent increase in the observed K_m value.

Evaluation of EAAT1 and EAAT2 mRNA expression in WT and PrPKO astrocytes

As the increased $V_{\max}$ values for transport could be due to alterations in transporter expression levels, EAAT1 and EAAT2 mRNA expression was analyzed by quantitative RT-PCR. In primary astrocyte cultures, EAAT1 mRNA expression was 16-fold higher than EAAT2 expression in both WT and PrPKO astrocytes (Figure 3). Treatment with dbcAMP increased expression of both EAAT1 and EAAT2 mRNA, but EAAT1 remained higher than EAAT2 in both WT and PrPKO astrocytes (Figure 3). Astrocytes were also co-cultured with neurons for 14 days, under conditions reported to induce an increase in EAAT2 expression as well as other morphological and biochemical changes reminiscent of astrocytes *in vivo* (Schlag et al., 1998; Swanson et al., 1997; Yang et al., 2009). In these co-cultures, expression of both EAAT1 and EAAT2 mRNA was increased compared to primary astrocyte cultures. However, EAAT1 expression was still higher than EAAT2 expression, although in astrocyte-neuron co-cultures, the difference was only 3.7–4.9-fold due to the larger increase in EAAT2 relative to EAAT1 (Figure 3). No significant difference between EAAT mRNA expression in WT versus PrPKO astrocytes was detected.

Evaluation of EAAT1 and EAAT2 protein expression in WT and PrPKO astrocytes

EAAT 1 and EAAT2 protein levels were also analyzed in WT and PrPKO astrocytes cultured under the same conditions described above, as well as in astrocytes cultured with neuron-conditioned medium (NCM). To eliminate multimerization following cell lysis, additional astrocyte cultures were lysed in buffer containing 1.0% SDS and analyzed by immunoblot for EAAT1 and EAAT2 expression. EAAT1 protein expression was increased in both WT and PrPKO astrocytes co-cultured with neurons (NCC) or NCM relative to controls (Figure 4A), and there was a small increase (22%) in EAAT1 in PrPKO versus WT astrocytes under both NCC and NCM conditions (Figure 3 4B) In contrast, EAAT2 protein expression was extremely low in all cultured astrocytes relative to mouse brain controls (data not shown). Thus, these EAAT protein expression data were in agreement with the mRNA expression data shown above (Figure 3).

Evaluation of EAAT 1 and EAAT2 activity

To assess the contribution of EAAT1 and EAAT2 to the total observed transport of D-aspartate in WT and PrPKO astrocyte cultures, transport of 5 μ M D-aspartate was measured in the presence of inhibitors that preferentially act on EAAT1 or EAAT2. Inhibitors were

added at concentrations estimated to block about 80% of the targeted EAAT with minimal activity at the other subtype (Bridges and Esslinger, 2005). In all of the cultures, activity was markedly reduced in the presence of the EAAT1-preferring inhibitor, LSOS. Remaining activity was 16–32% of control values (Table 2), indicating that EAAT1 accounted for at least 68–84% of transport detected. Consistent with these results, in the presence of the EAAT2 selective inhibitor, DHK, the remaining activity was 73–89% (Table 2), indicating that EAAT2 accounted for much less of the total activity. Thus, the majority of D-aspartate transport observed under all four culture conditions was mediated by EAAT1, which was consistent with our mRNA data (Figure 3). Therefore the quantitative differences in D-aspartate transport detected in WT and PrPKO astrocytes (Figure 2 and Table 1) appeared to be mainly attributable to changes in EAAT1 activity. Low levels of EAAT2 expression, coupled with the potency and specificity of the inhibitors, precluded a definitive conclusion as to possible changes in EAAT2 activity.

Comparison of sensitivity of WT and PrPKO neurons to glutamate-mediated excitotoxicity

Inadequate clearance of L-glutamate by astrocytes can lead to excitotoxic neuronal death through excessive activation of ionotropic excitatory amino acid receptors, especially the NMDA subtype (Choi 1992; Rosenberg and Aizenman 1989; Speliotis et al. 1994). Therefore, we tested the ability of WT and PrPKO astrocytes to protect neurons from L-glutamate-mediated excitotoxicity *in vitro*. The excitotoxic vulnerability of neurons and the co-cultures of astrocytes and neurons were determined after 14 days in culture. Surviving neurons were quantified 24 hours later based on MAP2 immunoreactivity (Shin et al. 2005). Compared to untreated controls, exposure to 20 μ M L-glutamate caused an insignificant reduction in the number of both WT and PrPKO neurons (Figure 5A and 5B), with no observable changes in morphology (not shown). In contrast, a 10 min exposure to 30 μ M or 40 μ M L-glutamate significantly reduced the survival of neurons in the WT cultures compared to neurons in the PrPKO cultures (Figure 6A). Furthermore, the surviving neurons exhibited a decreased number of neurites and altered neurite morphology (Figure 5C-F). At 50 μ M L-glutamate, both WT and PrPKO cultures showed similar damage (Figure 5G and 5H). Thus, the increased EAAT activity observed in the PrPKO cultures correlated with an increased resistance to damage by a limited range of L-glutamate concentrations (30 μ M and 40 μ M) compared to WT cultures.

To further examine the protective properties of the PrPKO astrocytes, WT neurons were grown on a bed of purified PrPKO astrocytes in the presence of 5 μ M cytosine arabinoside to prevent growth of WT astrocytes accompanying the neurons. After exposure to 30 μ M or 40 μ M L-glutamate, WT neurons exhibited an increased survival when cultured with PrPKO astrocytes compared to WT astrocytes (Figure 6A). The levels of survival were similar to those observed when PrPKO neurons were cultured with PrPKO astrocytes (Figure 6A). Thus PrPKO astrocytes were able to uniformly lessen the extent of excitotoxic neuronal loss in both WT and PrPKO co-cultures and this decreased vulnerability correlated with an increased presence and activity of EAAT1 (Figures 3 and 4, and Table 2). PrPKO neurons co-cultured with WT astrocytes also had a high percent of surviving neurons (Figure 6A) possibly due to PrPKO astrocytes seeded together with PrPKO neurons which may have survived the exposure to cytosine arabinoside in sufficient quantity to account for this unexpected protection.

Previously, PrPKO neurons were reported to be more vulnerable to excitotoxicity than WT neurons, when toxicity was induced by NMDA (Khosravani et al. 2008). This was opposite to our results using L-glutamate as the excitotoxic agonist. To study these differences further, we tested the vulnerability of our cultures to NMDA. When 1mM NMDA was added in place of L-glutamate, PrPKO neurons had a lower survival than did WT neurons (Figure 6B), consistent with Khosravani et al. The differences we found between

excitotoxicity induced by L-glutamate and NMDA appeared to be due to the ability of astrocytes to remove L-glutamate, but not NMDA, from the culture media.

Discussion

In our kinetic and pharmacological characterization of primary astrocyte cultures, we observed increased EAAT activity in cells from PrPKO mice compared to PrP expressing WT mice. This finding was observed in astrocytes cultured both in the absence and presence of dbcAMP. Furthermore, increased transport by PrPKO astrocytes was shown to be functionally significant as PrPKO astrocytes protected both PrPKO and WT neurons from L-glutamate-mediated excitotoxicity to a greater extent than did WT astrocytes. Levels of L-glutamate are believed to reach millimolar levels in the synaptic cleft during excitatory transmission (Clements et al. 1992) and are rapidly cleared to markedly lower (0.1–1 μ M) homeostatic levels (Herman and Jahr 2007). Therefore, the differences in $V_{\max}$ observed between PrPKO and WT astrocytes could have a marked influence on transmitter clearance and glutamatergic signaling.

Kinetic and physiological data showed that PrPKO astrocytes exhibit greater L-glutamate transport than WT astrocytes, although the underlying mechanisms responsible remain unclear. The increased EAAT activity in PrPKO versus WT astrocytes was accompanied by only a modest elevation in EAAT1 expression in PrPKO astrocytes, suggesting the alterations could be due to indirect mechanisms that effect surface localization and/or changes in intrinsic activity. L-Glutamate transporters are subject to interactions with modulating proteins (Jackson et al. 2001) and to post-translational modifications that can result in altered transporter function (Adolph et al. 2007; Duan et al. 1999; Lin et al. 2001; Munir et al. 2000, Schlag et al. 1998). The localization of PrP and EAATs to lipid rafts may provide an environment conducive to direct and/or indirect interactions between these proteins (Butchbach et al. 2004; Naslavsky et al. 1997).

In our in vitro cultured astrocytes the effect of the PrPKO genotype on glutamate transport activity appeared to be mediated primarily through an action on EAAT1. Even in astrocyte-neuron co-cultures where EAAT2 expression was highest, mRNA expression of EAAT1 was still approximately 4-fold higher than EAAT2 (Figure 3), and transport experiments in the presence of EAAT inhibitors could not clearly demonstrate a significant functional contribution by EAAT2 (Table 2) (Schlag et al., 1998). Further studies will be required to determine whether PrP can also influence expression of EAAT2 in various situations in vivo. However, even if the effect of PrP expression is limited solely to EAAT1, this influence is of significant interest because this subtype has important roles in certain areas including cerebellum, inner ear, circumventricular organs and retina (for reviews see Danbolt 2001 and Rauen et al. 1999). For example, lack of EAAT1 in knockout mice has been found to be associated with reduced motor coordination and increased sensitivity to cerebellar traumatic injury (Watase et al. 1998) as well as increased susceptibility to noise-induced injury (Hakuba et al. 2000). Furthermore, human patients with mutant EAAT1 were found to have clinical deficits including ataxia, hemiplegia, and seizures (Jen et al. 2005).

In contrast to our findings, a previous study reported that PrPKO astrocytes transported less L-glutamate than did WT astrocytes (Brown and Mohn 1999). However, this study used ^3H -L-glutamate as a substrate, rather than ^3H -D-aspartate, and the observed decrease was based on determinations at a single substrate concentration (100 μ M), making direct comparisons difficult. The most notable difference between the two studies, however, concerns the genetics of the mouse strains used to generate the cell cultures. In the Brown and Mohn study, the PrP WT mice were (129/SvEv x C57BL/6J) F1 hybrids, while PrPKO mice were homozygous for a random mixture of 129/SvEv and C57BL/6J genes. Thus, the background

genes of these PrPKO mice differed completely from the WT mice used. In contrast, in our experiments the PrPKO mice were backcrossed nine times to C57BL/10SnJ mice, the WT strain in this study. Therefore, in our PrPKO mice, only the PrP gene (*Prnp*) itself and areas immediately adjacent to this gene were still of strain 129/Ola origin. It is possible that these adjacent genes might also contribute to the differences observed in our experiments.

Astrocytic EAATs mediate the fine balance between having sufficient L-glutamate in the synapse for neuronal signaling without exceeding the threshold that would trigger excitotoxic pathology. To test if the increased transport exhibited by PrPKO astrocytes was physiologically relevant, we studied the toxicity of L-glutamate on mixed cortical cultures, where differences in transport capacity have previously been shown to modulate susceptibility to excitotoxicity (Robinson et al. 1993; Rosenberg and Aizenman 1989; Rothstein et al. 1996). The decreased neuronal damage observed within a narrow range of L-glutamate concentrations in cultures containing PrPKO astrocytes (Figure 6A) suggested that enhanced glutamate transport by PrPKO astrocytes lessened the excitotoxic insult.

However, this protection from L-glutamate-mediated excitotoxicity was distinct from the increased neuronal vulnerability of PrPKO neurons to NMDA-mediated excitotoxicity. As observed previously by others (Khosravani et al. 2008), we found that PrPKO neurons were intrinsically more vulnerable to NMDA-mediated excitotoxic injury than WT neurons. The difference between the two insults lies not with the action of L-glutamate and NMDA as NMDA receptor agonists, but with the fact that the EAATs do not transport NMDA. Thus, when NMDA was added to the mixed neuron-astrocyte co-cultures (Figure 6B), the experiment reflected the direct effect of NMDA on PrPKO and WT neurons. In contrast, when L-glutamate was added (Figure 6A), astrocytic transport of L-glutamate was able to reduce the excitotoxic challenge. Thus, increased transport of L-glutamate by PrPKO astrocytes appeared to be effective in protecting PrPKO neurons from death despite an increased vulnerability to excitotoxic injury.

Increased clearance of L-glutamate by astrocytes *in vivo* may contribute to the neurophysiological abnormalities observed previously in PrPKO mice. For example, reduced excitatory post-synaptic potentials (Carleton et al. 2001), impaired formation of long-term potentiation (Collinge et al. 1994; Criado et al. 2005; Manson et al. 1995), reductions in after-hyperpolarization potentials (Mallucci et al. 2002; Ratte et al. 2002) and abnormal responses to NMDA antagonist MK-801 (Coitinho et al. 2002) observed in PrPKO mice all suggest attenuation of L-glutamate-mediated signaling. Many of these alterations would be consistent with the premature termination of the L-glutamate signal and/or the excessive clearance of L-glutamate from the extracellular space surrounding EAA receptors. Consistent with such a conclusion, the EAATs have been shown to regulate the extracellular levels of L-glutamate available to activate synaptic and extrasynaptic receptors in specific excitatory circuits (Diamond 2005; Dzubay and Otis 2002; Huang et al. 2004; Turecek and Trussell 2000). Thus, the impact of alterations in EAAT activity may be greatest in those synaptic connections ensheathed by astrocytes, where the presence and positioning of the transporters has been shown to modulate glutamatergic neurotransmission (Anderson and Swanson 2000; Bridges and Esslinger 2005; Eulenburg and Gomeza 2010).

Low EAAT activity has been reported in a number of neurodegenerative diseases, including amyotrophic lateral sclerosis (Rothstein et al. 1992), HIV-associated dementia (Sardar et al. 1999), and Alzheimer's disease (Masliah et al. 1996). Increased levels of glutamate in the synaptic cleft may lead to neuronal death through excitotoxicity (Beart and O'Shea 2007). Accordingly, enhancement of L-glutamate transport has been regarded as a potential therapeutic goal. However, hyperactive EAAT activity and consequent reduced NMDA receptor signaling is not without its own complications, as has been suggested to be the case

in schizophrenia (Miyamoto et al. 2005). Future therapeutic approaches to modulating EAAT activity in any disease will require targeting a level of transporter activity that will maintain an optimal level of L-glutamate in the synaptic cleft, balancing physiological and pathological signaling during all phases of neuronal activity.

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Figure 1. Comparison of PrP expression on primary astrocytes harvested from WT and PrPKO mice

A. Live primary astrocytes from WT and PrPKO mice were labeled with anti-PrP monoclonal antibody, D13 (green), fixed, permeabilized and labeled with anti-GFAP, specific for astrocytes (red). Primary antibodies were visualized following incubation with Alexa Fluor-conjugated secondary antibodies as described in Methods. Nuclei were stained with DAPI. Results show strong PrP staining on the cell surface of WT astrocytes and no PrP staining on PrPKO astrocytes.

B. Study of surface PrP expression on WT and PrPKO primary astrocytes by flow cytometry. Live astrocytes were labeled with primary antibody, D13. PrP immunoreactivity was measured by FACS following fixation and incubation with an Alexa Fluor-conjugated secondary antibody as described in Methods. Graph shows cell frequency plotted versus fluorescence intensity. WT astrocytes showed strong cell surface PrP staining and PrPKO astrocytes showed no detectable PrP staining.

Figure 2. Comparison of D-aspartate transport by EAATs in WT and PrPKO astrocytes

A. EAAT activity was measured in primary astrocytes cultured from WT and PrPKO neonatal mice. Astrocytes were incubated for 5 minutes with various concentrations of D-aspartate, a non-metabolized analog of L-glutamate. Transport rate at each concentration was measured (solid and open circles) and then fit to the Michaelis-Menten equation using non-linear regression (solid and dashed curves). Results show faster transport by PrPKO astrocytes.

B. EAAT activity was measured in primary astrocytes after 10 days exposure to dbcAMP. As expected, transport rates increased in both WT and PrPKO astrocytes (note the scale bar difference between Figure 2A and 2B). Results show faster transport by PrPKO astrocytes.

C. The V_{max} of EAAT-mediated transport for each experiment examining primary astrocytes 7 days post-harvest is shown. WT astrocytes (circles) and PrPKO astrocytes (squares). Results show higher V_{max} values in PrP KO astrocytes.

D. Similar to 2C, the V_{max} of EAAT-mediated transport for each experiment examining primary astrocytes treated with dbcAMP is shown. Results show higher V_{max} values in dbcAMP-treated PrPKO astrocytes compared to WT astrocytes.

Figure 3. Analysis of EAAT1 and EAAT2 mRNA in WT and PrPKO astrocytes by real-time RT-PCR

EAAT1 and EAAT2 mRNA levels were quantified in WT and PrPKO (KO) astrocytes 7 days following purification (alone) or following an additional 10 day treatment with 0.25mM dibutyryl cAMP (dbcAMP) or after 14 days of neuron co-culture (neurons) as described in Methods. Values shown are average relative expression of four cultures where results were normalized to mouse β actin. EAAT1 expression was higher than EAAT2 expression under all culture conditions, although the difference between EAAT1 and EAAT2 was lower in neuronal co-cultures. However, in no comparison did PrPKO and WT astrocytes differ from each other significantly in EAAT1 or EAAT2 mRNA expression.

Figure 4. Analysis of EAAT1 protein in cultured wild type and knockout PrP astrocytes by immunoblotting

Cultured astrocytes from wild type (WT) and knockout (KO) PrP mice were harvested in buffer containing 1% SDS, and 10 ug of protein from each sample was separated by 10% Tris-Glycine SDS-PAGE with immunoblotting to detect EAAT1 (panel A, dimer at ~98 kDa) as described Methods. A representative immunoblot is shown in panel A.

Immunoreactive bands from two independent SDS-PAGE gels were analyzed using the Li-Cor Odyssey imaging system to determine alterations in Integrated Density Values (panel B). WT and KO astrocyte cultures were grown in neurobasal medium (NBM), were co-cultured with neurons (NCC), or treated with neuron-conditioned media (NCM). Results demonstrated that EAAT1 was highly induced by NCC or NCM in both WT and KO astrocytes relative to NBM conditions (panel A). Further, EAAT1 was slightly elevated in KO astrocyte cultures relative to WT astrocyte cultures (panel B, Gels 1 and 2).

Figure 5. Neuronal sensitivity to L-glutamate-mediated excitotoxicity

Neuronal cultures derived from WT or PrPKO mice (gestational period 14–16d) were plated onto a bed of purified WT or PrPKO astrocytes, respectively, and co-cultured for 14 days as described in Methods. On day 14, coinciding with NMDA receptor expression, co-cultures were exposed to 20μM (A-B), 30μM (C-D), 40μM (E-F) and 50μM (G-H) L-glutamate for 10 minutes. 24 hours following exposure, co-cultures were fixed, permeabilized, and stained with anti-MAP2 to identify neurons. Nuclei were visualized with DAPI. WT neurons showed more damage than PrPKO neurons at glutamate concentrations of 30 and 40μM.

Figure 6. Increased astrocytic protection against L-glutamate-mediated excitotoxicity by PrP KO astrocytes

A. Percent of MAP-2-positive WT or PrPKO neurons in neuron/astrocyte co-cultures 24 hours following a ten minute L-glutamate exposure (30 μ M or 40 μ M). The data (mean $\pm$ SEM) were obtained from four independent co-culture experiments. Results show increased toxicity in cultures with WT neurons co-cultured with WT astrocytes compared to cultures with PrPKO neurons and PrPKO astrocytes. Cultures of WT neurons and PrPKO astrocytes showed significantly less toxicity, suggesting that increased glutamate uptake by PrPKO astrocytes could provide a protective effect for WT neurons. Experiments in which PrPKO neurons were cultured with WT astrocytes also showed less excitotoxicity which could possibly be the result of contaminating PrPKO astrocytes from preparations of PrPKO neurons.

B. Percent of MAP-2 positive neurons in neuron/astrocytes co-cultures 24 hours following a ten minute exposure to NMDA. The data (mean $\pm$ SEM) were obtained from three independent co-culture experiments as described in panel A. In the presence of NMDA, increased neurotoxicity was seen in PrPKO co-cultures compared to WT co-cultures.

Kinetics of D-aspartate transport in WT and PrPKO astrocytes

Genotype	Treatment	n	K_m ¹	V_{max} ²	Fold Change ³
WT	None	13	40 ± 8	407 ± 29	1.7 ± 0.19 x
KO	None	9	63 ± 19	687 ± 61 ⁴	
WT	dbcAMP	11	66 ± 10	697 ± 57	2.5 ± 0.2 x
KO	dbcAMP	9	173 ± 29 ⁶	1768 ± 100 ⁵	

Data from Figure 2 were fit by non-linear regression to the Michaelis-Menten equation. n is the number of independent observations. V_{max} and K_m values are mean ± SEM.

¹ μ M

² pmol/min/mg protein

³ Fold increase in V_{max}

⁴ p = 0.0007, comparison of V_{max} in WT vs. PrPKO astrocytes. All statistics were done using non-parametric Mann-Whitney u-test.

⁵ p = 0.0005, comparison of V_{max} in dbcAMP-treated WT vs. PrPKO astrocytes

⁶ p = 0.002, comparison of K_m in dbcAMP-treated WT vs. PrPKO astrocytes.

Effect of EAAT1 and EAAT2 inhibitors on D-aspartate transport by WT and PrPKO astrocytes cultured in various conditions

Table 2

		% transport activity remaining in the presence of EAAT Inhibitors ¹			
Genotype	Treatment ²	D-asp transport ³ (pmol/min/mg)	LSOS (EAAT1)	DHK (EAAT2)	TBOA (EAAT 1-5)
WT	None	30 ± 2	18 ± 4 %	76 ± 11 %	7 ± 4 %
	None	31 ± 3	20 ± 4 %	89 ± 8 %	8 ± 4 %
PrPKO	None	31 ± 3	20 ± 4 %	89 ± 8 %	8 ± 4 %
	None	31 ± 3	20 ± 4 %	89 ± 8 %	8 ± 4 %
WT	dbcAMP	39 ± 7	30 ± 6 %	82 ± 6 %	11 ± 3 %
	dbcAMP	38 ± 2	26 ± 3 %	73 ± 13 %	9 ± 3 %
PrPKO	dbcAMP	38 ± 2	26 ± 3 %	73 ± 13 %	9 ± 3 %
	dbcAMP	38 ± 2	26 ± 3 %	73 ± 13 %	9 ± 3 %
WT	NCM	23 ± 2	16 ± 1 %	73 ± 4 %	3 ± 1 %
	NCM	23 ± 2	27 ± 2 %	88 ± 11 %	8 ± 1 %
PrPKO	NCM	23 ± 2	27 ± 2 %	88 ± 11 %	8 ± 1 %
	NCM	23 ± 2	27 ± 2 %	88 ± 11 %	8 ± 1 %
WT	Neurons	25 ± 0	32 ± 1 %	77 ± 5 %	6 ± 2 %
	Neurons	27 ± 0	20 ± 1 %	73 ± 8 %	5 ± 2 %
PrPKO	Neurons	25 ± 0	32 ± 1 %	77 ± 5 %	6 ± 2 %
	Neurons	27 ± 0	20 ± 1 %	73 ± 8 %	5 ± 2 %

¹Na⁺ dependent transport of 5μM D-aspartate was measured in the presence of 500μM LSOS (EAAT1 inhibitor), 300μM DHK (EAAT2 inhibitor) or 500μM TBOA (inhibitor of EAAT and EAAT2). Activity remaining (as % control) is shown as mean ± SEM of four independent observations.

²WT and PrPKO were cultured for 7 days as described in Methods (no treatment). To increase expression of EAAT2 astrocytes were cultured in the presence of dbcAMP or NCM or co-cultured in the presence of neurons, as described in Methods.

³Transport was measured at a concentration of 5μM D-aspartate.