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Influence of Prion Protein Expression on Function of Excitatory Amino Acid Transporters in Mouse Primary Astrocytes

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INFLUENCE OF PRION PROTEIN EXPRESSION ON FUNCTION OF EXCITATORY AMINO ACID TRANSPORTERS IN MOUSE PRIMARY ASTROCYTES

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Influence of prion protein expression on function of excitatory amino acid transporters in mouse primary astrocytes.

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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 (PrP KO) mice than in astrocytes from C57BL/10SnJ wildtype (WT) mice. Experiments using EAAT subtype-specific inhibitors demonstrated that in both WT and PrP KO astrocytes, the majority of transport was mediated by EAAT1. Furthermore, PrP KO astrocytes were more effective than WT astrocytes at alleviating L-glutamate-mediated excitotoxic damage in both WT and PrP KO neuronal cultures. Thus, in this model, PrP KO astrocytes exerted a functional influence on neuronal survival and may therefore influence regulation of glutamatergic neurotransmission *in vivo*.

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CHAPTER ONE

INTRODUCTION

Prion protein (PrP) is expressed in several mammalian tissues and is highly expressed in the central nervous system. While the precise function of PrP is unknown, studies suggest that PrP plays a role in many aspects of neurotransmission mediated by the neurotransmitter L-glutamate. Glutamatergic neurotransmission is dependent on L-glutamate availability in the synaptic cleft and subsequent activation of L-glutamate receptors. Specificity of activity is regulated by excitatory amino acid transporters (EAATs) expressed on neurons and astrocytes. The potential role PrP plays in neurotransmission and the pivotal role of EAATs in controlling L-glutamatergic neurotransmission provided the rationale to studying whether a function of PrP is to regulate concentrations of L-glutamate through modulation of EAAT activity.

1.1 Prion Protein

Prion Protein (PrP) is an evolutionarily conserved protein expressed in a number of different species including fish, amphibians, birds, cervids, ungulates, rodents and primates (Aguzzi et al., 2008). The gene encoding PrP is located on chromosome 2 in *mus musculus* (Prnp) and chromosome 20 in *homo sapiens* (PRNP). In some species, including bovine, deer, elk, mouse, rat and sheep, the Prnp gene contains 3 exons, while in other species including hamsters and humans, the gene contains 2 exons. Uniquely, the entire reading frame is contained in one exon (Basler et al., 1986), which in mice is exon 3. Gene expression is controlled by sequences contained in the 5'-flanking region, first intron and 3'-untranslated sequences.

Similar to other membrane proteins, biosynthesis of PrP begins in the rough ER and traffics through the Golgi to its final location on the plasma membrane. In the rough ER, PrP is subject to post-translational modifications including removal of its N-terminal signal peptide, addition of a C-terminal glycosyl-phosphatidylinositol (GPI) anchor, addition of N-linked glycosylations

and formation of a disulfide bond. PrP then passes through the Golgi for further modifications before reaching the plasma membrane where it exists as a double glycosylated protein anchored to the plasma membrane in detergent- resistant lipid rafts via the GPI anchor.

PrP exists as a disordered amino terminal flexible domain (amino acid residues 23-124) and globular carboxyl terminal domain (amino acid residues 125-226). The N-terminal domain contains a highly conserved octapeptide repeat region that is able to bind copper with μM affinity. The C-terminal domain contains three alpha helices (amino acid residues 144-154, 173-194 and 200-220) and an anti-parallel beta sheet formed by beta strands 128-131 and 161-164. A disulfide bond links cysteine residues 179 and 214.

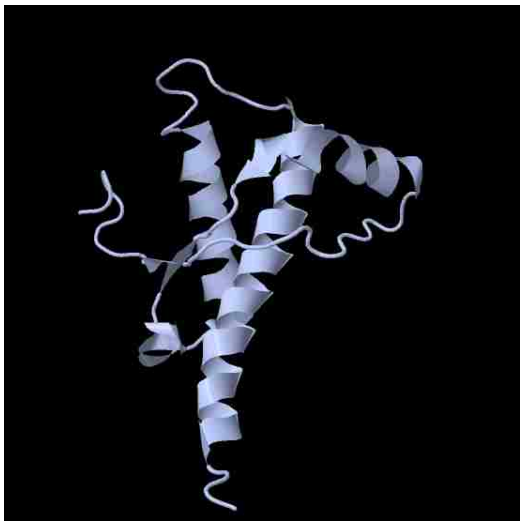

Figure 1.1 Prion Protein. a) NMR solution structure of elk PrP residues 121-231 (adapted from (Gossert et al., 2005)).

While the majority of PrP molecules are at the cell surface, a pool are constitutively cycling between the plasma membrane and endocytic compartments. Surface iodination of PrP revealed that a population of PrP molecules cycle between the plasma membrane and endocytic compartments with a transit time of 60 minutes (Shyng et al., 1993). However the mechanism of endocytosis is controversial. Immunogold EM revealed PrP enriched in caveolae at the plasma membrane of CHO cells (Peters et al., 2003) suggesting

a mechanism involving a caveolae-mediated endosomal pathway. However, stronger evidence exists suggesting PrP endocytosis is clathrin-mediated, which is unusual for GPI anchored proteins that lack an intracellular sequence necessary for interaction with endocytic adaptor proteins. Electron microscopy revealing PrP in clathrin-coated vesicles, prevention of internalization by disrupting clathrin lattices (Shyng et al., 1994) and studies using cells transfected with dynamin mutant K44A (Magalhaes et al., 2002) strongly suggest clathrin is involved in endocytosis. Internalization of GPI anchored PrP may involve usage of its positively charged domain (KKRPKP) in the amino terminal region to interact with the extracellular region of an integral membrane protein that also contains a localization signal in its cytoplasmic domain for clathrin-mediated endocytosis.

While GPI anchored proteins have many diverse functions, a definitive understanding of the physiological function of PrP is still unknown. With the intent of understanding the function of PrP, two independent lines of PrP knockout mice were generated. The lines differed in the strategy used to disrupt Prnp gene expression: in *Zurich* $PrP^{0/0}$ mice, a portion of exon 3 of the Prnp gene was replaced with a neomycin phosphotransferase (*neo*) gene (Bueler et al., 1992) and in *Edinburgh* $PrP^{-/-}$ mice, a *neo* gene was inserted into exon 3 of the Prnp gene (Manson et al., 1994). In both lines of mice, there was a lack of overt disturbances in development or behavior (Bueler et al., 1992; Manson et al., 1994). Though PrP KO mice did not obviously suggest a function for PrP, they did provide definitive evidence that expression of PrP was necessary for productive infection with transmissible spongiform encephalopathies (Bueler et al., 1993; Sailer et al., 1994).

Further examination of PrP knockout mice revealed several subtle abnormalities suggestive of varied physiological functions (Table 1). However, many of the functional studies compared PrP KO mice to PrP expressing mice with different genetic backgrounds. Both *Zurich* $PrP^{0/0}$ and *Edinburgh* $PrP^{-/-}$ mice were constructed using a sub-strain of 129 embryonic stem cells. However, to maintain the PrP knockout phenotype, many subsequent lines were crossed to

different strains of mice resulting in a mixed background. Thus, in several studies, the possibility exists that the phenotype is a result of other genes whose alleles differ between the knockout and wild-type mice compared. The only studies to compare mice of the same genetic background (*Edinburgh* $PrP^{-/-}$ versus 129/Ola mice) demonstrated PrP KO mice to have an abnormal quantity and morphology of mitochondria (Miele et al., 2002), altered regulation of amyloid beta production in the brain (Parkin et al., 2007), altered oxidative homeostasis in the brain (Wong et al., 2001) and an inability to induce long term potentiation (LTP) in the hippocampus (Manson et al., 1995).

Phenotype of PrP KO mice[1]	Implied Function of PrP	Background Strains of Mice Compared [2,3]	Citation
Did not have LTP deficits when exposed to amyloid-β oligomers	Amyloid-β oligomer receptor	$PrP^{-/-}$ bc C57BL.6 vs. C57 BL/6 $PrP^{0/0}$ hybrid vs. C57BL6 "hybrid"	Lauren et al., 2009 Balducci et al., 2010 [4]
Chronic demylenating polyneuropathy [5]	Myelin maintenance	$PrP^{0/0}$ bc Balb/c vs. Balb/c	Bremer et al.,
Increased susceptibility to kainate induced seizures Altered expression of glutamate and GABA receptor subunits	Maintaining neuronal homeostasis	$PrP^{0/0}$ bc C57BL/6 vs. C57BL/6	Rangel et al., 2009
Inefficient iron transport. Decreased iron content in red blood cells	Iron uptake and transport	$PrP^{0/0}$ bc FVB vs. FVB	Singh et al., 2009
Increased lung colonization of immortalized $PrP^{0/0}$ mesenchymal embryonic cells	Control of metastasis formation	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Muras et al., 2009
Reduced induction of T-helper cell cytokines Poor control of autoimmune and infectious diseases	Late T-cell activation antigen	$PrP^{0/0}$ bc FVB/N vs. FVB/N	Ingram et al., 2009
Reduced olfactory detection [5] Reduced paired pulse plasticity at dendrodendritic synapses [5]	Influences processing of sensory information by the olfactory system	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J) $PrP^{-/-}$ vs. 129/Ola	Le Pichon et al., 2009
Increased anxiety and poor performance in social recognition asks following brain injury influencing cortical development	Aids in recovery following brain injury Influences cortical development relating to short term memory	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Xikota et al., 2008
Slower rate of astrocyte maturation Reduced neuritogenesis	Receptor for stress inducible protein Mediates astrocyte development Modulates neuron-glia crosstalk	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J) $PrP^{-/-}$ bc C57BL/10 vs., $Prnp^{wt/wt}$	Arantes et al., 2009 Caetano et al., 2008 Lima et al., 2007
Delayed feedback inhibition of hypothalamic-pituitary-adrenal (HPA) axis following acute stress	Regulation of HPA axis	$PrP^{-/-}$ vs. 129/Ola	Sanchez-Alavez et al., 2008
Abnormal functioning and lack of LTP formation in cerebellar granule cells Poor performance in motor control tasks	Granule cell development	$PrP^{0/0}$ mixed vs. F_2 (129S1/SvImJ X C57BL/6J)	Prestori et al., 2008
Decreased defense responses when confronted with coral snakes	Modulation of innate fear and panic related behaviors	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Lobao-Soares et al., 2008

Phenotype of PrP KO mice[1]	Implied Function of PrP	Background Strains of Mice Compared [2,3]	Citation
Higher activity of neutral and acid sphingomyelinase	Regulation of sphingolipid associated signaling	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Schmalzbauer et al., 2008
Increased and prolonged NMDA evoked currents in hippocampal neurons Increased susceptibility to NMDA mediated neuronal death	Inhibitor of NR2D subunit of NMDA receptor	Littermates of $PrP^{0/0}$ X 129 or FVB)	Khosravani et al., 2008
Decreased infiltration of inflammatory cells and activated microglia following encephalomyocarditis virus infection Increased neuronal apoptosis	Role in induction of inflammation and inhibitor of apoptosis	$PrP^{0/0}$ mixed vs. F_1 ($Prnp^{0/+}$ X $Prnp^{0/+}$)	Nasu-Nishimura et al., 2008
Weaker afterhyperpolarizations in cererbellar and hippocampal neurons due to stronger calcium buffering and extrusion rates	Role in neuronal calcium homeostasis	$PrP^{0/0}$ bc FVB/N vs. FVB/N	Powell et al., 2008
Increased mitochondrial respiration and free radical production Defects in mitochondrial morphology Altered superoxide dismutase activities	Protection against oxidative stress	$PrP^{-/-}$ vs. 129/Ola	Paterson et al., 2008 Lobao-Soares et al., 2005[4]
Altered dorsal root ganglion axonal growth (substrate dependent)	Interacts with vitronectin	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J) $PrP^{-/-}$ bc C57BL/10 vs., $Prnp^{wt/wt}$	Hajj et al., 2007
Resistant to thermal and visceral inflammatory nociception	Role in nocicpetive transmission mediated by inflammation	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Meotti et al., 2007
Decreased $[Ca^{2+}]_I$ levels Decreased neuronal viability to H_2O_2	H_2O_2 sensor	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Krebs et al., 2007
Higher percentage of $CD8^+$ spleen dendritic cells	Influences development of $CD8^-$ dendritic cells	$PrP^{0/0}$ bc C57BL/6 vs. C57Bl/6	Martínez del Hoyo G, 2006
Decreased Ca^{2+} influx via L-type voltage gated Ca^{2+} channels	Ca^{2+} homeostasis	Littermates of C57Bl6 x 129Sv Prnp+/- mice	Fuhrmann et al., 2006
Reduced allogenic T Cell response	Role in immunological synapse between dendritic cells and T cells	$PrP^{0/0}$ bc C57BL/6 vs. C57Bl/6	Ballerini et al., 2006

Phenotype of PrP KO mice[1]	Implied Function of PrP	Background Strains of Mice Compared [2,3]	Citation
Increased infarct volume following transient or permanent ischemia Reduced Akt activation Enhanced caspase 3 activation	Anti-apoptotic	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Weise et al., 2006
Delay in neural differentiation	Role in neurogenesis and adult neural development	$PrP^{-/-}$ bc C57BL.6 vs. C57 BL/6	Steele et al., 2006
Impaired self renewal of hematopoietic stem cells	Supports self renewal of hematopoietic stem cells	$PrP^{-/-}$ bc four or six times to C57 BL/6J CD45.2 vs. CD45.2 C57BL/6	Zhang et al., 2006
Arrested NCAM dependent neurite outgrowth	Nervous system development using NCAM as a signaling receptor	$PrP^{0/0}$ bc C57BL/6 vs. C57Bl/6	Santuccione et al., 2005
Impaired hippocampal dependent spatial learning Impaired LTP in dentate gyrus	Regulator of glutamatergic signaling	$PrP^{-/-}$ bc two times to C57BL.6 vs. C57 BL/6 $PrP^{-/-}$ vs. 129/Ola	Criado et al., 2005[5]
Reduced copper content in synaptosomes	Regulates copper concentrations in synaptosomes	$PrP^{0/0}$ hybrid vs. C57BL6 and 129 “hybrid”	Giese et al., 2005
Increased male susceptibility to ischemia	Neuroprotection against ischemia	$PrP^{0/0}$ hybrid vs. C57BL/6	Sakurai-Yamashita et al., 2005
Decreased anxiety and locomotion in response to acute stress	Modulation of anxiety and muscle activity in response to stress	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Nico et al., 2005
Increased infarct size (200% after ischemia, increased Erk1/2, STAT I and caspase 3	Neuroprotective, anti-apoptotic	$PrP^{0/0}$ mixed vs. F_1 (129/Sv X C57BL/6J)	Spudich et al., 2005

[1] Phenotype of PrP KO mice or primary cells harvested from PrP KO mice
[2] $PrP^{0/0}$ = Zurich PrP KO mice on a mixed background. $PrP^{-/-}$ = Edinburgh PrP KO mice on a 129/Ola background
[3] bc = backcross to the wild-type strain (as indicated) for at least eight generations
[4] Disputed results or conclusions of the study
[5] Phenotypic rescue in PrP expressing transgenic lines of mice

A number of studies, albeit using mice of different backgrounds, have also reported synaptic abnormalities relating to L-glutamatergic neurotransmission. PrP KO mice suffer from deficits in hippocampal spatial memory (Criado et al., 2005), have abnormal responses to NMDA receptor antagonist MK-801(Coitinho et al., 2002) and exhibit reduced excitatory post-synaptic potentials (Carleton et al., 2001) and afterhyperpolarization potentials (Mallucci et al., 2002). Decreased levels of LTP in PrP KO mice has been rescued by expression either of human PrP (Whittington et al., 1995; Asante et al., 2004) or by neuronal expression of mouse PrP (Criado et al., 2005). Primary neurons cultured from PrP KO mice exhibit increased NMDA receptor activation and an increased vulnerability to NMDA mediated excitotoxicity (Khosravani et al., 2008). Collectively, these observations suggest that PrP KO mice suffer from deficits in neurotransmission, consistent with attenuated glutamate signaling, which could result from excessive clearance of L-glutamate from the synaptic space.

1.2 The Glutamatergic System

L-glutamate is the primary excitatory neurotransmitter in the mammalian nervous system, mediating neuronal communication that includes fast synaptic transmission and higher order processing necessary for development and learning and memory. L-glutamate is sequestered intracellularly maintaining an intracellular-extracellular concentration gradient of several million-fold (Zerangue and Kavanaugh, 1996; Herman and Jahr, 2007). Thus, preserving homeostatic levels of L-glutamate is important in maintaining specificity of signaling and appropriate receptor activation.

The highest concentration of L-glutamate in the CNS is found in nerve terminals (Ottersen et al., 1992) . Here, L-glutamate is packaged into synaptic vesicles by proton-dependent vesicular transporter, V-GLUT. Once an action potential reaches the nerve terminal, vesicles loaded with L-glutamate fuse with the plasma membrane and release their contents extracellularly. In the synaptic space, L-glutamate can activate G-protein coupled metabotropic L-glutamate

receptors and ionotropic L-glutamate receptors. Diffusion of L-glutamate also permits activation of receptors extrasynaptically.

Metabotropic L-glutamate receptors are G-protein coupled receptors whose activation play a modulatory role by regulating the production of intracellular messengers and consequently activating or inhibiting second messenger systems. The receptors belong to Class C G-protein coupled receptors, characterized by 7 transmembrane helices connected by 3 intracellular and 3 extracellular loops. To date, there have been 8 metabotropic glutamate receptors cloned that are divided into 3 groups. Group I includes mGlu1 and mGlu5 that are localized post-synaptically on neurons and who are coupled to phosplipase C. Group II includes mGlu2 and mGlu3, found both pre- and post-synaptically and are negatively coupled to adenylyl cyclase. Group III includes mGlu4, mGlu6, mGlu7 and mGlu8 that are localized pre-synaptically. The synaptic location of these receptors allows them to modulate both neurotransmitter release and regulation of fast acting ionotropic L-glutamate receptors.

Fast-acting ionotropic L-glutamate receptors are L-glutamate gated ion channels that permit an influx of sodium and/or calcium from the extracellular environment to the inside of the cell. Binding of L-glutamate to low affinity AMPA or Kainate receptors (who also preferentially bind AMPA and kainate, respectively) causes an influx of sodium, which serves to depolarize the cell. In contrast, the high affinity NMDA receptor acts as a coincidence detector, requiring glycine and L-glutamate binding, as well as previous depolarization of the membrane in order to permit the entrance of sodium and calcium into the cell. Compared to AMPA and kainate receptors, NMDA receptors have slow deactivation times and are critical in mediating higher order processing.

Several distinct subtype compositions have been identified differing in their sensitivities to pharmacological agents, their channel opening times, their distribution in the brain and their relative concentrations during development. Most NMDA receptors are heteromeric molecules consisting of two obligatory NR1 subunits and two NR2 subunits. Four isoforms of the NR2 subunit have

been identified, NR2A, NR2B, NR2C and NR2D. These isoforms are distinct in determining characteristics of the NMDA receptor, which influence channel kinetics. The type of NR2 subunit present determines deactivation times of the receptor in response to L-glutamate. The deactivation times span a 50-fold range with NR2A subunits deactivating fastest and NR2D subunits slowest (NR2A > NR2C = NR2B >> NR2D). NMDA receptor subunit composition and subsequent receptor deactivation kinetics reflect the nature of the synapse in which they are expressed. NR2A containing receptors are present at most mature synapses while NR2B containing receptors are expressed predominantly during development. The changes in the molecular makeup of NMDARs are part of what defines the “critical period” during development in which neuronal circuits are fine tuned (Cull-Candy and Leszkiewicz, 2004).

L-glutamate is the major excitatory neurotransmitter in the CNS and as such, extracellular levels of L-glutamate must be tightly controlled to maintain specificity of signaling. Depending on the location of the synapse, L-glutamate signaling has various functions throughout the CNS such as mediating experience dependent synaptic plasticity through the phenomena of long-term potentiation (LTP) and long-term depression (LTD), development and fine-tuning of excitatory synapses, the perception of pain and modulation of signaling by other neurotransmitters such as dopamine. The concentration of L-glutamate in the extracellular space and the amount of time it remains in the space determines the number of L-glutamate receptors activated. Therefore, keeping extracellular concentrations of L-glutamate low allows for a high signal-to-noise ratio in synaptic and extrasynaptic transmission (Danbolt 2001).

Furthermore, keeping the extracellular concentration of L-glutamate low prevents neuronal death. Excessive activation of L-glutamate receptors and prolonged excitatory synaptic transmission can lead to neuronal death through excitotoxicity (Olney 1996). L-glutamate mediated excitotoxicity is mediated by a sustained activation of L-glutamate receptors. Because of its high permeability to calcium, the NMDA receptor in particular mediates the majority of excitotoxic insult although AMPA and Kainate receptors play a role in sustained

depolarization of neurons (Salinska et al., 2005; Waxman and Lynch 2005). The molecular events leading to neuronal death are unclear although altered calcium homeostasis plays a distinctive role in dysregulation of signal transduction pathways, activation of calcium dependent calpains (Wu et al., 2004), and activation of metabolic pathways that generate free radicals (Salinska et al., 2005). Neuronal death follows excitotoxicity and therapeutic strategies aimed against excitotoxcity are hoped to delay pathogenesis of many neurodegerative diseases.

Thus, maintaining basal concentrations of L-glutamate is critical in maintaining signal specificity and preventing neuronal death. Levels of L-glutamate following vesicular release are believed to reach millimolar levels during excitatory transmission (Clements 1992) but must be rapidly cleared to markedly lower (0.1-1μM) homeostatic levels (Herman and Jahr 2007). Extracellular L-glutamate concentrations are regulated by the activity of excitatory amino acid transporters present on neurons and astrocytes.

1.3 Excitatory Amino Acid Transporters

Unlike other neurotransmitters, such as acetylcholine whose extracellular concentrations are controlled by enzymatic degradation, rapid termination of L-glutamate signaling is achieved by cellular uptake. The majority of L-glutamate transport is mediated by high affinity, Na^+ dependent excitatory amino acid transporters (EAATs). To date, five EAAT subtypes with high affinity for L-glutamate have been identified. EAAT1 (GLAST) and EAAT2 (GLT-1) are primarily glial transporters responsible for the bulk of L-glutamate clearance. EAAT3 (EAAC), EAAT4 and EAAT5 are expressed by neurons.
Levels of transporter subtypes are regionally, developmentally and functionally specific.

Characterization of the EAATs was aided by molecular pharmacology, used to both distinguish between subtypes and understand the transport process. Experiments aimed at understanding basic amino acid substrate selectivity identified D-aspartate as an inhibitor of L-glutamate uptake and

excellent alternate substrate with a K_m in glial cultures of 60-80μM compared to L-glutamate whose K_m is 30-90μM (reviewed in Bridges et al., 1999). One of the earliest competitive inhibitors characterized, effective at all five EAATs and a substrate of EAAT1-4, was B-threo-hydroxyaspartate (ß-THA). Subsequently, ß-THA has been used as a backbone for the development of other EAAT inhibitors incuding ß-*threo*-benzyloxyaspartate (TBOA), a non-substrate pan-EAAT inhibitor with K_i values in the 1-10μM range.

To date, the majority of inhibitors exhibiting a high degree of selectivity for one transporter subtype over the others are limited to EAAT2. Potent inhibitors of EAAT2 include the classic inhibitor dihydrokainate (DHK, K_i = 23μM) and, more recently, WAY213613 (IC_{50} = 85nM) (Bridges et al., 1999; Dunlop et al., 2005). Alternate substrates at EAAT1, 4-MG and L-serine-O-sulfate can be used to distinguish EAAT 1 from EAAT2 activity. Recently, a selective EAAT1 inhibitor, UCPH 101, has been developed (Jensen et al., 2009). The usage of pharmacology, either through subtype selective inhibitors or through subtype specific alternate substrates, has allowed understanding the delineation of subtype-specific roles in the transport of L-glutamate.

Glial transporters, EAAT1 and EAAT2 are expressed throughout the mature CNS and are responsible for the majority of L-glutamate clearance. However, there is developmental and regional enrichment of one subtype over the other. During development, EAAT1 is the dominant transporter expressed and functional though protein levels of both transporters increase dramatically during synaptogenesis (Shibata et al., 1996; Sutherland et al., 1996). In the adult however, EAAT1 expression and function dominates in Bergmann glia of the cerebellum. In other regions of the brain, EAAT2 is the major glutamate transporter functional though EAAT1 expression remains high (Danbolt, 2001). Whether the same astrocyte expresses both EAAT1 and EAAT2 is controversial; immunogold labeling demonstrated co-expression (Haugeto et al., 1996) while analysis of promoter activity demonstrated a non-overlapping pattern of expression (Regan et al., 2007). The importance of the relative stoichiometry of one subtype over the other in a single astrocyte or region is unknown. EAAT2

knockout mice do suffer from lethal spontaneous seizures (Tanaka et al., 1997). However, levels of neuronal death following selective pharmacological inactivation of EAAT2 in adult mice did not mirror that when all glutamate transporters were rendered inactive (Selkirk et al., 2005), suggesting that following stress, EAAT1 can rapidly be mobilized for dominant function.

In contrast to glial L-glutamate transporters, neuronal transporters are considered to make a minor contribution to overall L-glutamate clearance. EAAT3 expression is highest in the hippocampus, cerebellum, basal ganglia, forebrain and spinal cord. EAAT4 expression is highest on Purkinje neurons in the cerebellum and EAAT5 expression is limited to the retina. EAAT3 and EAAT4 are expressed postsynaptically and are though to provide L-glutamate as a precursor for GABA synthesis (Sepkuty et al., 2002).

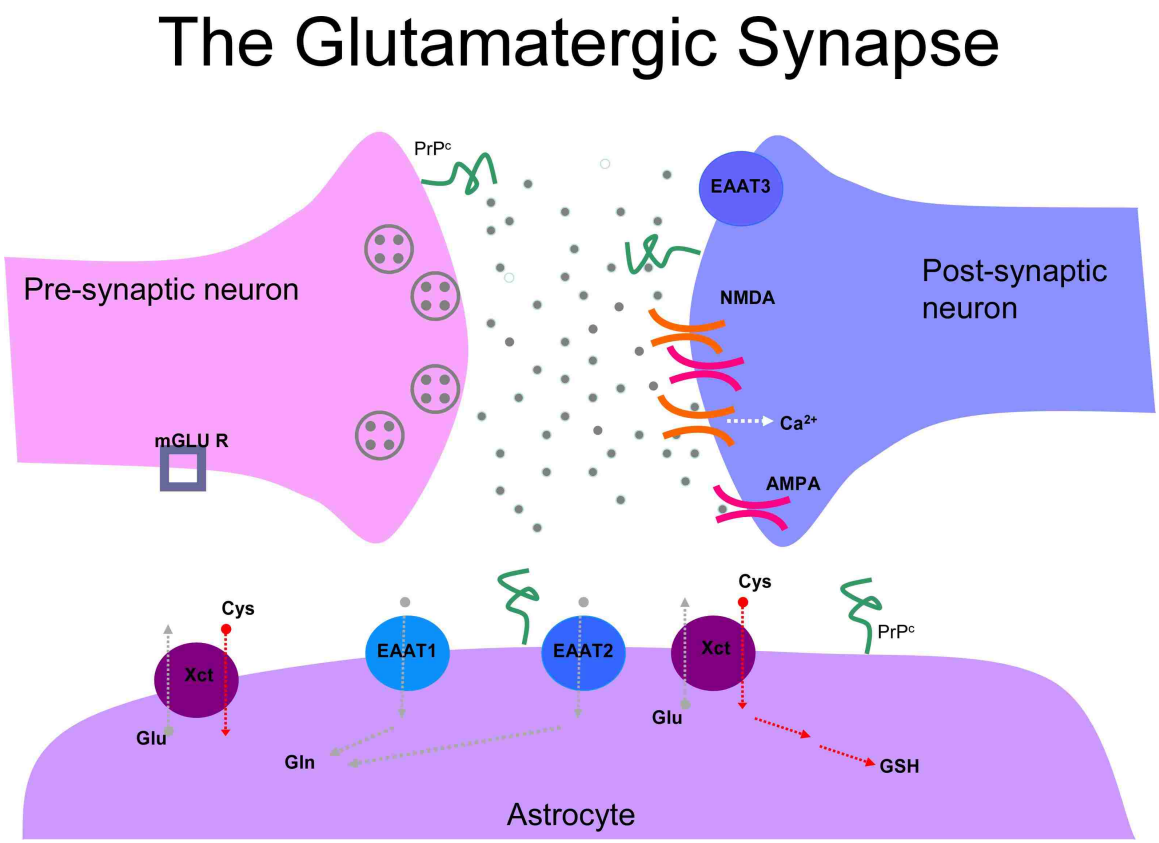

Figure 1.2 The Glutamatergic Synapse.

Following an action potential in the presynaptic neuron, L-glutamate vesicles fuse with the plasma membrane and release L-glutamate into the synaptic cleft. L-glutamate can bind to and activate NMDA, AMPA, Kainate and metabotropic L-glutamate receptors causing fast or long-lasting

changes in the activated neuron. The L-glutamate signal is terminated by transport of L-glutamate into astrocytes by EAAT1 and EAAT2. L-glutamate is then rapidly synthesized into glutamine (Gln) by glutamine synthetase. L-Glutamate is also transported by EAAT3 and Xct. EAAT 3 is localized to neurons and provides L- glutamate for GABA synthesis. System Xct, localized to astrocytes, exchanges glutamate for cysteine, which is subsequently used for gluthathione synthesis. PrP is expressed on astrocytes and neurons in the synapse.

The EAATs are defined as symporters, transporting the substrate by co-transport and counter-transport of ions (Bunch et al., 2009). The overall goal of transport is to accumulate L-glutamate intracellularly against its concentration gradient. This is powered by the co-transport of 3 Na^+ ions and one H^+ ion. Following release of co-transported L-glutamate and ions, one K^+ ion is counter-transported and released to the extracellular space (Zerangue and Kavanaugh, 1996). The EAATs also conduct a thermodynamically uncoupled Cl^- flux, thereby acting as a Cl^- channel (Wadiche et al., 1995a).

The transport cycle occurs by an alternating access mechanism (Kavanaugh, 1998; Yernool et al., 2004) or cow chute mechanism (personal communication, Terri Mavencamp) in which two major conformations of the EAATs are employed. The first conformation is an outward conformation where the substrate-binding site faces the synaptic space. The second conformation is inward, allowing the substrate-binding site to be accessible to the cytoplasm of the cell. The full cycle of transport begins with binding of the co-transported ions (3 Na^+, 1 H^+) and substrate to the transporter in the outward conformation. This leads to translocation of the transporter resulting in an inwards conformation. The co-transported ions and substrate are released and intracellular K^+ binds. Consequently, the substrate binding site reorients to the outside (Tzingounis and Wadiche, 2007). Possibly due to the steps required sequester L-glutamate intracellulary, the full cycle is relatively slow, estimated to be between 60-80ms (Wadiche et al., 1995b). Reconciling the slow transport cycle with the mM amounts of L-glutamate present in the synaptic cleft during vesicular release suggest that the EAATs initially function as L-glutamate buffers, terminating excitatory signaling by trapping L-glutamate (Diamond et al., 1997).

Much of the structure of the EAATs has been inferred following the elucidation of the crystal structure of a bacterial orthologue of the EAATs, a sodium-dependent Asp transporter from *Pyrococcus horikoshii*, Glt_{Ph} (Yernool et al., 2004). The membrane transporter is composed of a bowl shaped homomer with three independent subunits. Each subunit of the trimer is capable of a transport cycle. The monomer contains an intercellular N and C termini, eight transmembrane (TM) regions and two hairpin loops. The glutamate binding site is at the bottom of the bowl, between the 2 hairpin loops, which are hypothesized to act as gates to the binding site (Boudker et al., 2007). The EAATs are glycosylated in the hydrophilic loop between TM 3 and TM4 although the number of glycosylations in the region differ among the EAAT subtypes (Seal and Amara, 1999).

The overall purpose of the EAATs is to maintain homeostatic levels of L-glutamate. Too much L-glutamate can lead to neuronal death through initiation of excitotoxic cascades. The impact of glial protection from L-glutamate mediated excitotoxicity through EAAT activity has been demonstrated in primary cultures altering glia/neuron ratios and by pharmacological blockade of the EAATs (Rosenberg and Aizenman, 1989; Robinson et al., 1993; Rothstein et al., 1996). Several neurodegenerative diseases have implicated low EAAT activity as contributing to pathology, including amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1992), HIV-associated dementia (Sardar et al., 1999), and Alzheimer's Disease (Masliah et al., 1996). Thus, increasing activity has been a therapeutic target. In a mouse model of ALS, increased EAAT expression through administration of β-lactam antibiotics delayed neuronal loss (Rothstein et al., 2005). However, high EAAT activity can also decrease the amount of synaptic L-glutamate necessary for proper L-glutamatergic neurotransmission. Elevated L-glutamate transport has led to decreased LTP in the hippocampus (Filosa et al., 2009) and impaired hippocampal dependent learning (Carmona et al., 2009). Hyperactive EAAT activity might contribute to reduced L-glutamatergic signaling observed in schizophrenia (Miyamoto et al., 2005). Thus, the EAATs play a powerful role in carefully regulating activation of L-glutamate receptors, ensuring

enough L-glutamate can be present for signaling without having too much to initiate excitotoxic pathways. Dysfunction in EAAT activity may be one of the causes of disease in Transmissible Spongiform Encephalopathies.

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CHAPTER TWO

STUDY OF INFLUENCE OF PRION PROTEIN EXPRESSION ON FUNCTION OF EXCITATORY AMINO ACID TRANSPORTERS IN MOUSE PRIMARY ASTROCYTES

By

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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 (PrP KO) mice than in astrocytes from C57BL/10SnJ wildtype (WT) mice. Experiments using EAAT subtype-specific inhibitors demonstrated that in both WT and PrP KO astrocytes, the majority of transport was mediated by EAAT1. Furthermore, PrP KO astrocytes were more effective than WT astrocytes at alleviating L-glutamate-mediated excitotoxic damage in both WT and PrP KO neuronal cultures. Thus, in this model, PrP KO astrocytes exerted a functional influence on neuronal survival and may therefore influence regulation of glutamatergic neurotransmission *in vivo*.

Introduction

Prion protein (PrP) is an evolutionarily conserved, glycophosphatidylinositol-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 (PrP KO) 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, PrP KO 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 glutamatergic synapse (Collinge et al., 1994; Manson et al., 1994; Carleton et al., 2001; Mallucci et al., 2002; Criado et al., 2005; Khosravani et al., 2008). These dysfunctions in PrP KO 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 in close apposition to the neuropil 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 PrP KO 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 PrP KO mice containing 98.5% sequence identity to the C57BL/10SnJ genotype. We found that astrocytes from PrP KO 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 functional in both PrP KO 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 PrP KO mice.

Materials and Methods

Animals.

C57BL/10SnJ mice (WT) were purchased from Jackson Labs (Bar Harbor, ME). Homozygous PrP null (PrP KO) mice on the 129/Ola background (Manson et al., 1994) were backcrossed nine times to C57BL/10SnJ mice selecting for the PrP KO allele by PCR identification at each cross (Race et al., 2009). Single nucleotide polymorphism (SNP) analysis was performed on DNA from PrP KO 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% CO_2 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). Purified astrocyte cultures were harvested with trypsin. For D-aspartate uptake assays, astrocytes were reseeded at 1 x 10^5 cells/well in 12 well plates and maintained with fresh media every three days. In some experiments, astrocytes were assayed seven days post-seeding, upon reaching confluence. In other experiments, confluent 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. The inclusion of dbcAMP induces biochemical and morphological changes more representative of astrocytes *in vivo* including greater EAAT expression (Schlag et al., 1998). Consistent with other reports (Schlag et al., 1998), we found that treatment with dbcAMP significantly increased EAAT1 and EAAT2 expression as judged by analysis of mRNA by quantitative PCR (Figure 3).

Primary cortical neurons were prepared essentially as described by Kaech and Banker (Kaech and Banker, 2006). Briefly, whole cerebral neocortices removed from either WT or PrP KO 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). Cells, 5 x 10^4 cells/well, were plated onto a bed of 2.5 x 10^4 purified astrocytes that had been plated in 24 well plates two days earlier. Neuron cultures 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 (500µl) (Invitrogen, Carlsbad, CA) supplemented with Glutamax (Invitrogen, Carlsbad, CA), B-27 with antioxidants (Invitrogen, Carlsbad, CA) and 5µM 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. Neuronal cultures were used 14 days later, coinciding with NMDA receptor expression (Choi et al., 1987).

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 μg monoclonal antibody D13, specific for PrP, in 50μL PBS. Following 1 hour incubation at 37°C, cells were 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 μg Alexa Fluor488 goat anti-human IgG (Invitrogen, Carlsbad, CA) in 50μL PBS. Control tubes of WT and KO astrocytes were fixed, blocked and incubated with Alexa Fluor488 goat anti-human IgG alone. Data was collected by FACSCanto II flow cytometer (Becton Dickinson, San Jose CA) and 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 KH_2PO_4, 0.3 mM Na_2HPO_4, 1.1 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM HEPES, pH 7.4) and pre-incubated at 37 °C for 5 min. Transport rates were determined using ^{3}H-D-aspartate, which is effectively transported as an EAAT substrate, yet not metabolized by cells following uptake (Koch et al., 1999). Transport assays were carried out in which uptake was initiated by replacing the preincubation buffer with buffer containing ^{3}H-D-aspartate (5-300μM, 2-12μCi/ml). Following a five-minute incubation, 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 $\pm$ 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 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)), which were added simultaneously at the indicated concentrations with 5μM ^{3}H-D-aspartate.

Quantitative RT-PCR Analysis.

Purified astrocytes, 1 x 10^4, were seeded onto 24 well plates. Upon reaching confluence, some wells were treated with 0.25mM dbcAMP for an additional ten days. Confluent astrocytes were harvested with trypsin and lysed using Qiashredder (Qiagen, Valencia, CA). Neuron – astrocyte cocultures as described above were also harvested with trysin and lysed using Qiashredder after 14 days in vitro. 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 = $2^{-\Delta C_T}$

Immunoblotting

Purified WT astrocyte cultures were washed briefly in 5ml cold PBS. Astrocytes were lysed by shaking incubation with 1ml cold lysis buffer (5mM Tris HCl p.H 7.4, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 1X Protease Inhibitor Cocktail) at 4°C for 5 minutes. Cell lysates were placed in a pre-cooled 1.5ml centrifuge tube and centrifuged at 100*g* for 15 minutes. Supernatants were collected and stored at -20°C. Protein concentration was quantified using the bicinchoninic acid method (Pierce, Rockford,IL). cell lysates (10ug) were boiled in 1X Sample Buffer containing Reducing Agent (Invitrogen, Carlsbad, CA) for 3 minutes and loaded onto 4-12% Bis Tris gels (for EAAT1) and 12% Tris-Glycine gels (for EAAT2). 4-12% Bis-Tris gels, used for assessing monomeric and multimeric EAAT2 that runs at 64kDa and above, were run at 120 V for 4 hours in MOPS buffer. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane (37V, O/N). Membranes were blocked

in TBS with 0.1% tween 20 containing 5% milk. Membranes were probed with either antibodies specific for EAAT1 (1:2000, Tocris Cookson, St. Louis MO), or actin (1:10,000, Sigma Aldrich, St. Louis, MO) in TBS–tween for one hour at room temperature. Following three ten-minute washes with TBS-tween, membranes were probed with appropriate horseradish peroxidase conjugated secondary antibodies (EAAT 2 goat anti rabbit (1:3000); actin: goat anti-mouse (1:3000)) diluted in TBS tween for one hour at room temperature. Visualization was performed using chemiluminescence, according to manufacturer's recommendations (GE Healthcare Life Sciences, Pittsburg, PA).

L-Glutamate-mediated excitotoxicity.

At 14 days in vitro, neuron-glial co-cultures were exposed to varying concentrations of L-glutamate (10-50μM) in 500μl fresh Neurobasal (Invitrogen, Carlsbad, CA) media for ten minutes at room temperature. Following exposure, cells were gently rinsed with HBSS, after which half their original media (250μl) was added back to the well combined with 250μl fresh Neurobasal media with B27 supplement to maintain original culture conditions. Twenty-four hours following L-glutamate exposure, surviving neurons were enumerated. Cells were fixed with 3.7% formaldehyde for 15 minutes, washed with PBS, permeabilized with 0.1% Triton x100, 0.1% sodium citrate for ten minutes rinsed with PBS, and labeled with mouse anti- MAP2 (1:1000) (Millipore, Billerica, MA) to identify neurons followed by incubation 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. The surviving MAP2-positive neuronal cell bodies that colocalized with DAPI stained nuclei were quantified (Shin et al., 2005). Neuronal survival following L-glutamate exposure was expressed as percent survived relative to the total number of neurons counted in wells that had not been 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 PrP KO mice were used as negative controls. Cells were labeled with anti-GFAP, to identify astrocytes, and with a monoclonal antibody, D13, reactive with PrP. As expected, the majority of cells in both WT and PrP KO cultures were astrocytes and PrP immunoreactivity was only observed on WT astrocytes and not on PrP KO astrocytes (Figure 2-1A). Primary WT and PrP KO astrocytes were also examined by flow cytometry where live cells were labeled with D13. Surface expression of PrP was only observed on WT astrocytes (Figure 2-1B).

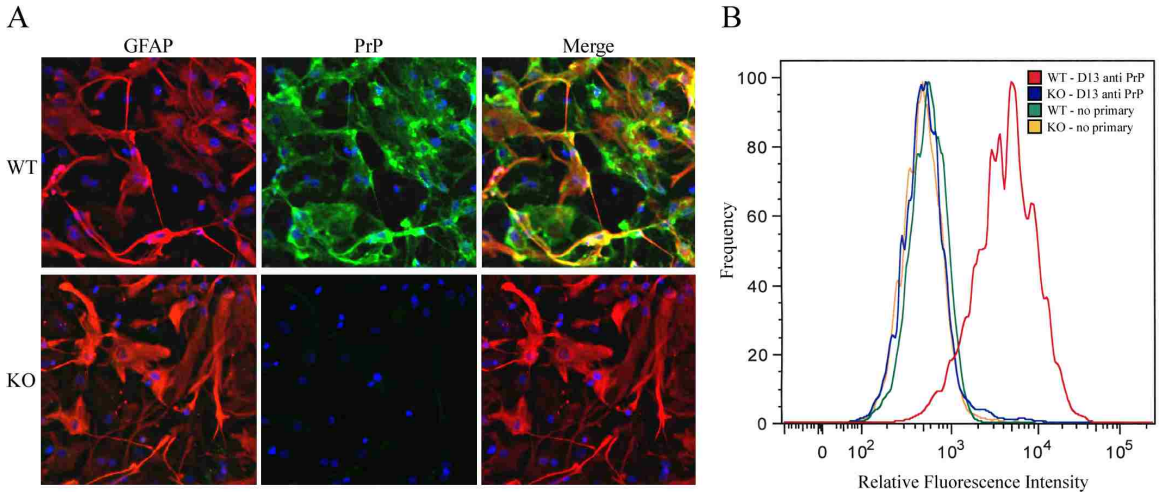


Figure 2-1

Comparison of PrP expression on primary astrocytes harvested from WT and PrP KO mice

A. Live primary astrocytes from WT and PrP KO 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 PrP KO primary astrocytes by flow cytometry. Live astrocytes were labeled with primary antibody, D13 anti-PrP. D13 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 KO astrocytes showed no detectable PrP staining.

Comparison of EAAT activity in WT and PrP KO astrocytes

We next examined the potential influence of PrP expression on L-glutamate homeostasis by analyzing EAAT–mediated transport by astrocytes prepared from WT and PrP KO mice. Both EAAT1 and EAAT2 are localized to astrocytes and are known to be responsible for the bulk of CNS glutamate transport (Danbolt, 2001). Sodium-dependent transport assays were performed using the non-metabolized EAAT substrate, D-aspartate, at a range of concentrations. As shown in Figure 2-2A, transport rates between WT and PrP KO astrocytes clearly diverged at concentrations of D-aspartate greater than 50μM. When fit to the Michaelis Menten equation, the V_{max} values were 1.7 fold higher in the PrP KO astrocytes compared to WT astrocytes (687 vs. 407 pmol/min/mg, Table 1-1 and Figure 2-2C).

Previous studies have demonstrated that 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) (Khelil et al., 1990; Le Prince et al., 1991; Miller et al., 1994; Jackson et al., 1995; Hosli et al., 1997; Swanson et al., 1997; Schlag et al., 1998; Daginakatte et al., 2008). Therefore, we also studied WT versus PrP KO astrocytes treated for ten days with dbcAMP (0.25mM). Initial experiments also confirmed that transport rates in the untreated cells were not significantly altered by the additional ten days in culture (data not shown). When dbcAMP-treated astrocytes were stained with D13 anti PrP, PrP immunoreactivity was only

observed in WT astrocytes (data not shown). In transport experiments, PrP KO 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 2-2B, Figure 2-2D, and Table 2-1). This increase was larger than the 1.7 fold increase observed in untreated astrocytes (Table 2-1). Thus, the highest V_{max} for D-aspartate transport were found in cells lacking PrP that had been treated with dbcAMP.

Though differences in V_{max} were observed between WT and PrP KO astrocytes both before and after treatment with dbcAMP, K_m values were only significantly different when astrocytes were cultured with dbcAMP (66 vs. 173μM, Table 2-1). Alterations in V_{max} values may reflect changes in either transporter activity or expression. However, the observed change in K_m between WT and PrP KO astrocytes following dbcAMP treatment suggest that factors associated with PrP may have a direct influence on transporter activity in the differentiated cells.

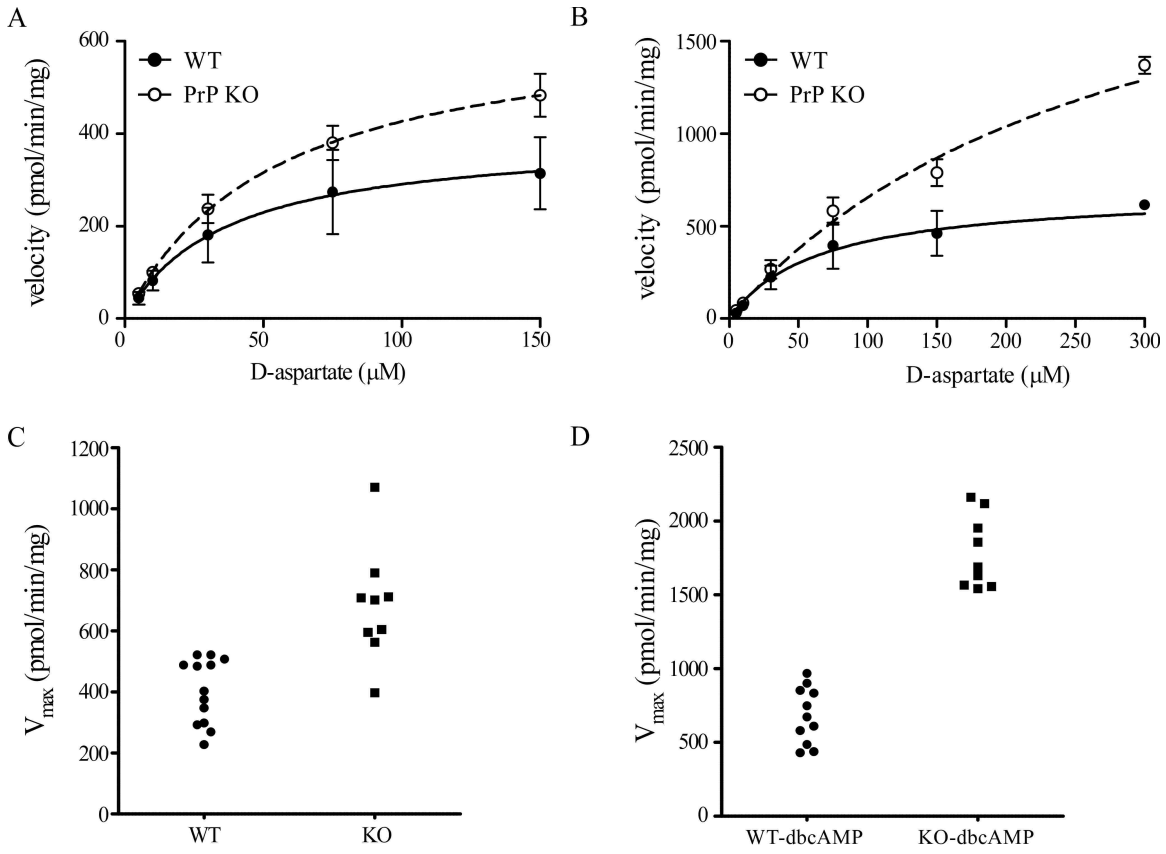


Figure 2-2

Comparison of D-aspartate transport by EAATs in WT and PrP KO astrocytes.

A. EAAT activity was measured in primary astrocytes derived from WT and PrP KO neonatal mice cultured 7 days post-harvest. Astrocytes were incubated with 5μM, 10μM, 30μM, 75μM or 150μM D-aspartate, a non-metabolized analog of L-glutamate, for 5 minutes. 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 are averages of data from multiple independent observations (N=13 for WT, N=9 for PrP KO). Results show faster transport by PrP KO astrocytes.

B. EAAT activity was measured in primary astrocytes, which were cultured in the presence of dbcAMP for an additional 10 days. This treatment induced morphological and biochemical changes in astrocytes including the increase in number of functional transporters. Astrocytes were incubated with 5μM, 10μM, 30μM, 75μM, 150μM or 300μM D-aspartate for 5 minutes. As expected, transport rates increased in both WT and PrP KO astrocytes (note the scale bar difference between Figure 2-2A and 2-2B). Transport rate at each concentration of D-aspartate (solid and open circles) was fit to the Michaelis Menten equation using non-linear regression (solid and dashed curves). Data are averages of multiple independent observations (N=11 for WT, N=9 for PrP KO). Results show faster transport by PrP KO astrocytes.

C. The V_{max} of EAAT-mediated transport for each experiment examining primary astrocytes 7 days post harvest is shown. WT astrocytes are respresented with solid circles and KO astrocytes represented as solid squares. Results show higher V_{max} values in PrP KO astrocytes.
D. Similar to 2-2C, the V_{max} of EAAT-mediated transport for each experiment is shown examining primary astrocytes treated with dbcAMP for ten additional days. Results show higher V_{max} values in dbcAMP-treated PrP KO astrocytes.

Table 2-1. Kinetics of D-aspartate transport in WT and PrP KO 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 2-2 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] µM

[2] pmol/min/mg protein

[3] Fold increase in V_{max}

[4] p = 0.0007, comparison of V_{max} in WT vs. PrP KO 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. PrP KO astrocytes

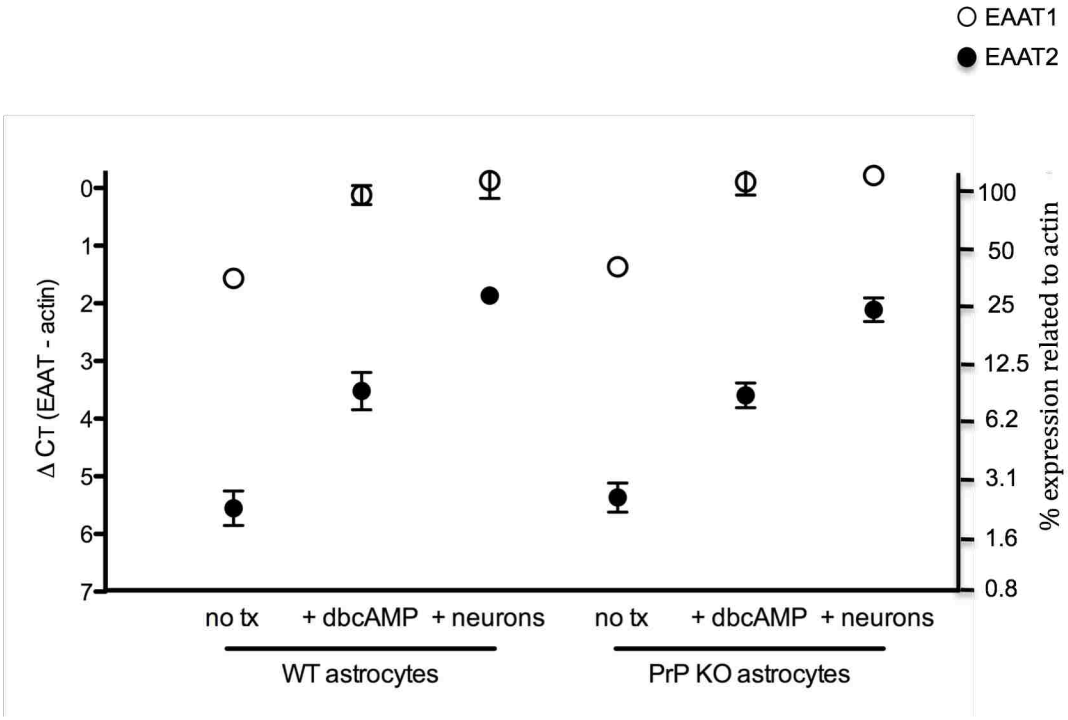
[6] p = 0.002, comparison of K_m in dbcAMP-treated WT vs. PrP KO astrocytes.

Evaluation of EAAT1 and EAAT2 expression and activity in WT and PrP KO astrocytes

The increased V_{max} values for transport observed in PrP KO astrocytes could be due to alterations in transporter expression levels. Quantitative RT-PCR was performed using mRNA from WT and PrP KO astrocytes. In untreated astrocytes, EAAT1 expression was approximately 16-fold higher than EAAT2 expression (30% vs. 2% of actin expression, Figure 2-3A). As expected, dbcAMP-treated astrocytes showed increased expression of both EAAT1 and EAAT2 mRNA (Schlag et al., 1998). In dbcAMP treated astrocytes, EAAT1 expression was approximately 12.5-fold higher than EAAT2 (100% vs. 7% of actin expression, Figure 2-3A). Therefore EAAT1 appeared to be the major transporter observed in both untreated and dbcAMP-treated astrocytes. In addition, EAAT1 mRNA levels were similar in PrP KO vs. WT astrocytes, and likewise, EAAT2 mRNA levels were similar in these same cells (Figure 2-3A). Therefore the increased transport observed in PrP KO astrocytes compared to WT astrocytes did not correlate with increased production of either EAAT1 or EAAT2 transporter mRNA.

Though dbcAMP treatment produces morphological and biochemical changes more representative of astrocytes *in vivo*, including an increase of both EAAT1 and EAAT2, co-culture with neurons can specifically induce EAAT2 expression (Gegelashvili et al., 1997; Swanson et al., 1997). Therefore, we also evaluated EAAT subtype expression in WT and PrP KO astrocyte-neuron co-cultures. Co-culture with neurons increased EAAT2 expression to 30% of actin expression, which was also detectable at the protein level (Figure 2-3B). However, EAAT1 expression was also increased in astrocyte-neuron co-cultures to 100% of actin expression, though upregulation did not differ from what was observed in dbcAMP astrocytes. Despite a 10-fold increase in EAAT2 expression specific to co-culture with neurons, however, EAAT1 mRNA expression was still higher than EAAT2 expression (100% vs. 30% of actin expression).

A.



B.

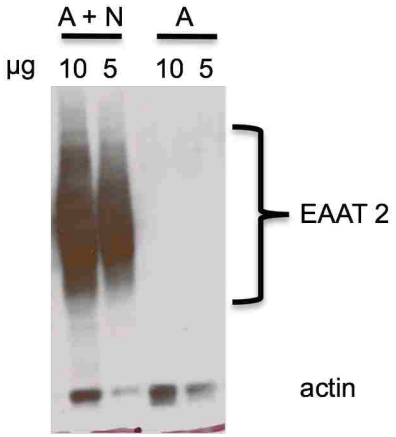


Figure 2-3 EAAT expression in WT and PrP KO astrocytes

A. Analysis of EAAT1 and EAAT2 in WT and PrP KO astrocytes by real time RT-PCR. EAAT1 and EAAT2 mRNA levels were quantified in WT and PrP KO (KO) astrocytes 7 days following purification (as defined in Methods), following an additional 10 day treatment with 0.25mM dbcAMP (as described in Methods) or following 14 day co-culture with neurons of the same genotype. Values shown are average $\Delta C_T \pm$ SEM of four - seven cultures where results were normalized to mouse ß actin. A low ΔC_T value corresponds to higher amount of transcript detected. Though dbcAMP treatment and co-culture with neurons increased EAAT2 expression,

EAAT1 expression was always higher in astrocyte cultures. In no comparison did PrP KO and WT astrocytes differ from each other significantly in EAAT mRNA expression.
B. Immunoblot of EAAT2 in WT astrocytes. EAAT2 immunoblot of lysates prepared from astrocytes 7 days following harvest (A) or following 14 day co-culture with WT neurons (A+N). EAAT 2 expression, which is undetectable in primary astrocytes, is upregulated when astrocytes are co-cultured with neurons.

To assess the contribution of individual EAAT subtypes to the total observed transport of D-aspartate in WT and PrP KO astrocytes, transport of 5μM D-aspartate was measured in the presence of inhibitors that preferentially act on individual EAAT subtypes (Bridges and Esslinger, 2005). In each of the cultures, activity was markedly reduced in the presence of EAAT1 selective inhibitor, LSOS (i.e. remaining activity was 20-30% of control measurements in the presence of 500μM LSOS, Table 2-2). Consistent with these results, the inclusion of the EAAT2 selective inhibitor, dihydrokainate (DHK, 300μM), produced much less inhibition and the remaining activity ranged from 73-89% of control (Table 2-2). Thus, the majority of uptake observed was mediated by EAAT1 as predicted by our mRNA expression data (Figure 3A and 3B). This was similar to previous reports of primary *in vitro* astrocyte cultures, including those treated with dbcAMP (Duan et al., 1999; Munir et al., 2000; Lin et al., 2001; Adolph et al., 2007).

Because of the marked increase in EAAT2 mRNA expression observed in astrocyte-neuron co-cultures, the pharmacological profile of WT and PrP KO co-cultures was also assessed. In all co-cultures, D-aspartate activity was markedly reduced in the presence of EAAT1 selective inhibitor, LSOS (i.e. remaining activity was 20-32% of control measurements, Table 2-2), while activity in the presence of EAAT2 selective inhibitor, DHK, produced much less inhibition (i.e. remaining activity was 73-77% of control measurements, Table 2-2). Thus, while EAAT2 mRNA and protein expression was increased in these co-cultures, its contribution to L-glutamate transport was masked by the high amounts of EAAT1 expression.

Table 2-2. D-aspartate transport in WT and PrP KO astrocytes in the presence of various EAAT inhibitors

			% activity retained 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 %
PrP KO	None	31 ± 3	20 ± 4 %	89 ± 8 %	8 ± 4 %
WT	dbcAMP	39 ± 7	30 ± 6 %	82 ± 6 %	11 ± 3 %
PrP KO	dbcAMP	38 ± 2	26 ± 3 %	73 ± 13 %	9 ± 3 %
WT	Neurons	25 ± 0	32 ± 1 %	77 ± 5 %	6 ± 2 %
PrP KO	Neurons	27 ± 0	20 ± 1 %	73 ± 8 %	5 ± 2 %

[1] Na^+ dependent transport of 5μM D-aspartate was measured in the presence of 500μM LSOS, 300μM DHK or 500μM TBOA. These blockers were included in the assays at concentrations estimated to block at least 80% of either EAAT1 or EAAT2 activity. TBOA was used as a control for maximal inhibition with specificity for both EAAT1 and EAAT2. Activity retained (% control) is shown as mean ± SEM of four independent observations.

[2] WT and PrP KO were cultured for 7 days as described in Method, treated for an additional ten days with 0.25mM dbcAMP to increase EAAT expression or were cultured in the presence of neurons, as described in Methods for 14 days.

[3] transport was measured at a concentration of 5μM D-aspartate.

Comparison of sensitivity of WT and PrP KO neurons to glutamate-mediated excitotoxicity

Numerous studies have concluded that 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 (Rosenberg and Aizenman, 1989; Choi, 1992; Speliotes et al., 1994). Therefore, we tested the ability of WT and PrP KO astrocytes to protect neurons of the same genotype from L-glutamate-mediated excitoxicity *in vitro*. After 14 days in culture, coinciding with NMDA receptor expression and the excitotoxic vulnerability of neurons, the co-cultures of astrocytes and neurons were exposed for ten minutes to L-glutamate at concentrations ranging from 10μM to 50μM. The number of surviving neurons was quantified based on MAP2 immunoreactivity 24 hours later as previously described by Shin et al (Shin et al., 2005). Compared to untreated controls, exposure to 20μM L-glutamate caused a slight reduction in the number of both WT and PrP KO neurons (Figure 2-4A and 2-4B), although the morphology of WT and PrP KO neurons remained similar to untreated controls (not shown). In contrast, exposure to 30μM and 40μM L-glutamate led to significantly reduced survival of neurons in the WT cultures compared to neurons in the PrP KO cultures (Figure 2-5A). Furthermore, the surviving neurons exhibited a decreased number of neurites and altered neurite morphology (Figure 2-4C-F). At 50μM L-glutamate, both WT and PrP KO cultures showed similar damage (Figure 2-4G and 2-4H). Thus, the increased EAAT activity observed in the PrP KO cultures correlated with an increased resistance to damage by a window (30μM and 40μM) of L-glutamate concentrations compared to WT neurons cultured with WT astrocytes.

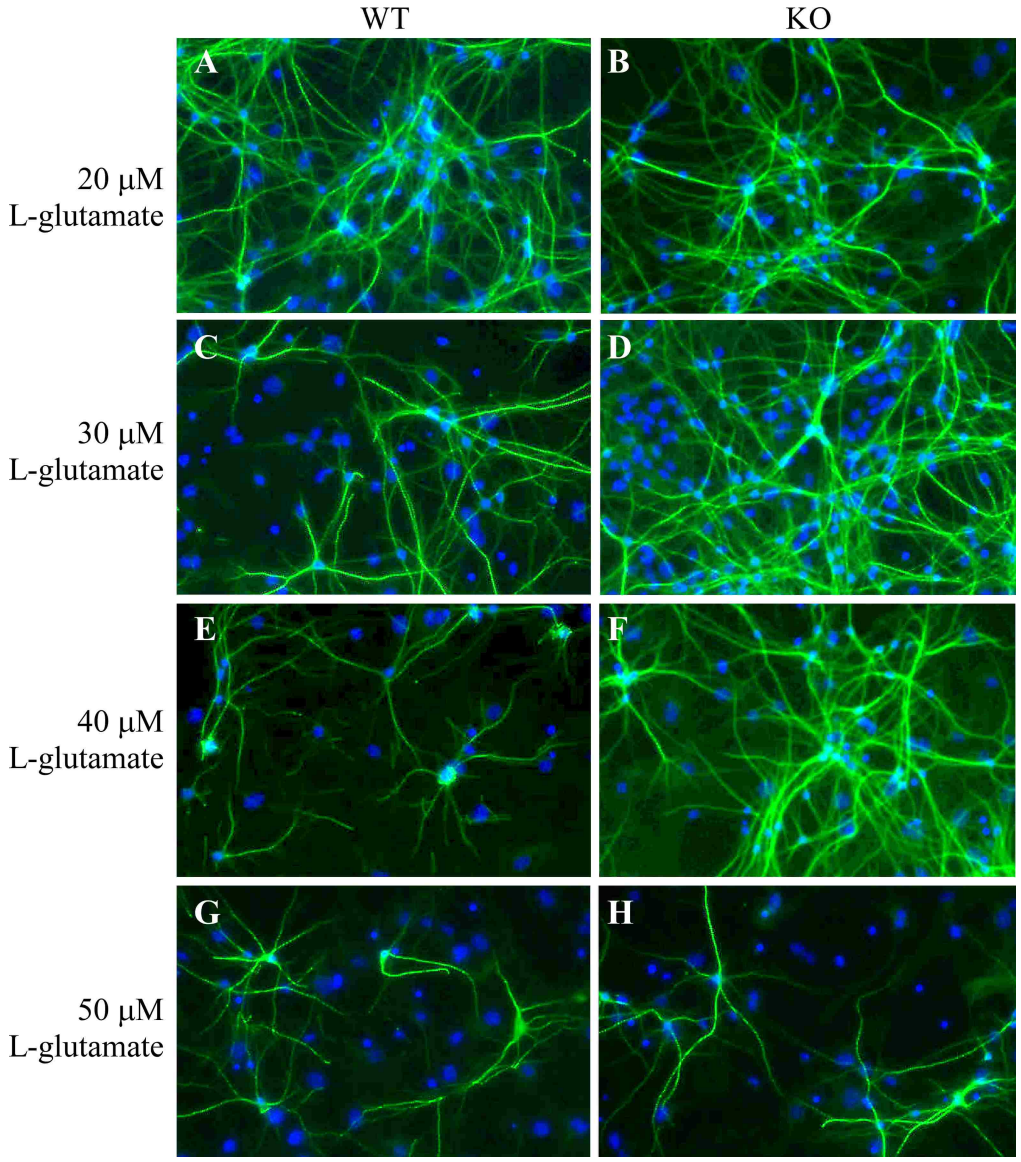


Figure 2-4

Neuronal sensitivity to L-glutamate-mediated excitotoxicity.

Neuronal cultures derived from WT or PrP KO mice (gestational period 14-16) were plated onto a bed of purified WT or PrP KO 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. MAP2 staining was visualized following incubation with an Alexa Fluor-conjugated secondary antibody. Nuclei were visualized with DAPI. WT neurons showed more damage than PrP KO neurons at glutamate concentrations of 30 and 40µM.

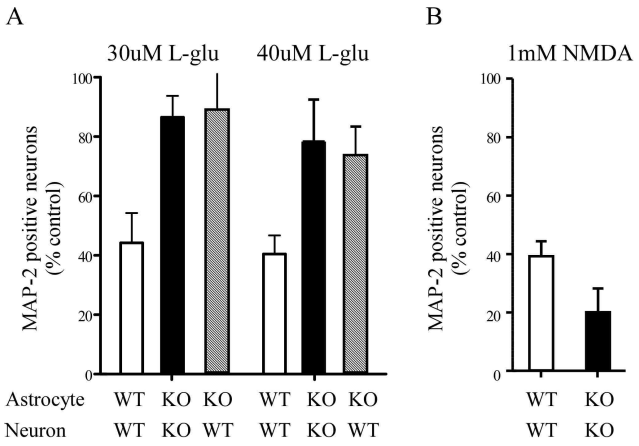


Figure 2-5

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

A. Percent of MAP-2-positive WT or PrP KO neurons in neuron/astrocyte co-cultures 24 hours following a ten minute L-glutamate exposure (30μM or 40μM). The data (mean ± SEM) were obtained from four independent co-culture experiments. Results show increased toxicity in cultures with WT neurons co-cultured with WT astrocytes. Cultures of WT neurons and PrP KO astrocytes showed significantly less toxicity. Experiments in which PrP KO neurons were plated on WT astrocytes yielded variable results (data not shown), which are most likely the result of contaminating PrP KO astrocytes from preparations of PrP KO 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 ± SEM) were obtained from three independent co-culture experiments as described in panel A. In the presence of NMDA, increased neurotoxicity was seen in PrP KO co-cultures compared to WT co-cultures.

We next examined if PrP KO astrocyte cultures could similarly provide increased protection to WT neurons. WT neurons were grown on a bed of purified PrP KO astrocytes in the presence of 5μM cytosine arabinoside (as described in Methods) to prevent WT astrocyte contamination and growth. After exposure to 30μM and 40μM, WT neurons exhibited a higher degree of survival when cultured on PrP KO astrocytes than when cultured on WT astrocytes (Figure 2-5A). The levels of survival were similar to those observed when PrP KO neurons were cultured with PrP KO astrocytes (Figure 2-5A). These data demonstrated that PrP KO astrocytes were able to uniformly lessen the extent of excitotoxic neuronal loss in both WT and PrP KO co-cultures.

A previous report showed that PrP KO neurons were more vulnerable to excitotoxicity than WT neurons when NMDA was used to induce the insult (Khosravani et al. 2008). This was opposite to the results we observed using L-glutamate as the excitotoxic agonist. To study whether these differences might be due to methodological differences or, more likely, to the inability of EAATs to clear NMDA from the extracellular space, we tested the vulnerability of our cultures to NMDA. When 1mM NMDA was added to our cultures in place of L-glutamate, KO neurons had a lower survival than did WT neurons (Figure 2-5B), consistent with Khosravani et al. Taken together, these results suggest that because L-glutamate is an EAAT substrate, the increased clearance by PrP KO astrocytes functioned to counteract the oversensitivity of PrP KO neurons to stimulation by excitatory receptor agonists.

Discussion

In our kinetic and pharmacological characterization of primary astrocyte cultures, we observed increased EAAT activity in cells from PrP KO mice compared to PrP expressing WT mice. This finding was observed in astrocytes cultured both in the absence and presence of dbcAMP, which has been shown to induce biochemical and morphological characteristics of astrocytes observed *in vivo* (Khelil et al., 1990; Le Prince et al., 1991; Miller et al., 1994; Jackson et al., 1995; Hosli et al., 1997; Swanson et al., 1997; Schlag et al., 1998; Daginakatte et al., 2008). Furthermore, increased transport by PrP KO astrocytes was shown to be functionally significant as KO astrocytes protected both KO 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 (0.1-1μM) homeostatic levels (Herman and Jahr 2007). Therefore, the differences in V_{max} observed between PrP KO and WT astrocytes could have a marked influence on transmitter clearance and glutamatergic signaling.

Though the kinetic and physiological data clearly suggests that PrP KO astrocytes have a greater capacity to transport L-glutamate, the underlying mechanisms for increased EAAT activity have been difficult to ascertain. We observed increased EAAT1 and EAAT2 activity in PrP KO versus WT astrocytes without increased expression of either transporter. Given the very high levels of EAAT protein present in these cultures, it has, however, been difficult to accurately correlate expression with activity (Schlag et al., 1998). As an alternative to higher total levels of EAAT protein, increased activity could be due to indirect mechanisms that affect surface expression of the transporters and/or changes in intrinsic activity, though we currently do not have data to differentiate between these processes. As opposed to the changes in Vmax, the difference in K_m between dbcAMP-treated WT and PrP KO astrocytes argues for a direct modulation of the transporters resulting in changed affinity for L-glutamate. L-Glutamate transporters are subject to interactions with modulating proteins (Jackson et al., 2001) and posttranslational 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). PrP expression may be involved in such modifications; we observed an increase in PrP mRNA expression following dbcAMP treatment in WT astrocytes. The localization of PrP and EAATs to lipid rafts may provide an environment conducive to interaction between these molecules (Butchbach et al. 2004; Naslavsky et al. 1997). As functional data indicate the majority of the transport quantified in the present experiments. Including astrocyte-neuron co-cultures, is mediated by EAAT1, it suggests that it is this subtype that is altered in the PrP KO astrocytes and leaves open the issues as to whether or not PrP expression may also influence EAAT2 and EAAT3.

In contrast to our findings, a previous study reported that cultures of astrocytes expressing PrP transported L-glutamate at a faster rate than did PrP KO astrocytes and that this difference was likely attributable to increases in K_m without a change in V_{max} (Brown and Mohn 1999). However, the decease in the rate of transport in the PrP KO astrocytes was only reported at a single

concentration (100 μM). A number of methodological differences, including the use of ^{3}H-L- glutamate as a substrate rather than ^{3}H-D-aspartate, determinations of background and specific uptake, as well as treating the cells with dbcAMP, make direct comparisons difficult. The most notable difference between the two studies, however, concerns the genetics of the strains used to generate the cell cultures. In the Brown and Mohn study, the PrP WT mice were (129/Sv(ev) x C57BL/6J) F1 hybrids, while PrP KO mice were homozygous for a random mixture of 129/Sv(ev) and C57BL/6J genes. Thus, the background genes of these PrP KO mice differed completely from the WT mice used. In contrast, the PrP KO mice used in our experiments were backcrossed nine times to C57BL/10SnJ mice, which was used as the WT for comparison. Therefore, in our PrP KO mice, only the Prnp (PrP) gene itself and areas immediately adjacent to the Prnp 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 PrP KO 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 (Rosenberg and Aizenman, 1989; Robinson et al., 1993; Rothstein et al., 1996). In our experiments, PrP KO astrocytes treated with dbcAMP exhibited the highest levels of transport (Table 2-1). Notably, co-culturing astrocytes with neurons induces many of the same morphological and biochemical changes induced by dbcAMP, including EAAT1 and EAAT2 expression (Gegelashvili et al., 1997; Schlag et al., 1998; Perego et al., 2000). When co-cultures contained PrP KO astrocytes, we found decreased evidence of excitotoxicity within a narrow range of L-glutamate concentrations (Figure 2-5A), suggesting that enhanced glutamate transport by PrP KO astrocytes, possibly at the rates seen in dbcAMP-treated PrP KO astrocyte cultures, lessened neuronal death.

However, this protection from L-glutamate-mediated excitotoxicity is distinct from the increased neuronal vulnerability of PrP KO neurons to NMDA-mediated excitotoxicity observed in our experiments (Figure 2-5B) and by others (Khosravani et al 2008). Exposure to NMDA demonstrated that PrP KO neurons are intrinsically more vulnerable than WT neurons to excitotoxic insults induced by excessive NMDA receptor activation. While L-glutamate and NMDA are both agonists at the NMDA receptor, L-glutamate transporters do not transport NMDA. Thus, when NMDA was added to the mixed neuron-astrocyte co-cultures (Figure 2-5B), the experiment reflected the direct effect of NMDA on PrP KO and WT neurons, as the EAATs could not transport NMDA and decrease its effective concentration. In contrast, when L-glutamate was added (Figure 2-5A), astrocytic transport of L-glutamate was able to alter its concentration, in agreement with the D-aspartate uptake measurements, and reduce the excitotoxic challenge. Unfortunately, the fragility of neurons precluded direct assays of transport capacity in the co-cultures. Thus, increased transport of L-glutamate by PrP KO astrocytes appeared to be effective in protecting PrP KO neurons from death despite an increased vulnerability to excitotoxic injury. Similar results were observed in the G93A SOD1 mouse model of amyotrophic lateral sclerosis where a drug-induced increase in EAAT activity (approximately 2-fold) was shown to be neuroprotective *in vitro* and delayed neuronal loss in vivo (Rothstein et al., 2005).

Increased clearance of L-glutamate by astrocytes *in vivo* may contribute to the neurophysiological abnormalities observed previously in PrP KO 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 afterhyperpolarization potentials (Mallucci et al., 2002) and abnormal responses to NMDA antagonist MK-801 (Coitinho et al., 2002) observed in PrP KO 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 (Turecek and Trussell, 2000; Dzubay and Otis, 2002; Huang et al., 2004; Diamond, 2005). 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). Similarly, the present work suggests that increased EAAT activity in the PrP KO mouse may contribute to its neurophysiological phenotype. Taken together, therapeutic approaches 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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CHAPTER THREE

INFLUENCE OF PRION PROTEIN MEMBRANE LOCALIZATION ON EXCITATORY AMINO ACID TRANSPORTER ACTIVITY

By

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ABSTRACT

Though the precise function of Prion protein (PrP) is unknown, numerous studies suggest a role in glutamatergic neurotransmission. In chapter two, we showed that PrP influenced L-glutamate signaling *in vitro* by impacting its clearance from the vicinity of neurons. Therefore, we investigated whether similar localization of PrP and excitatory amino acid transporters (EAATs) to lipid rafts influenced astrocytic EAAT activity. Astrocytes were harvested from C57BL10 (WT) mice, PrP knockout (PrP KO) mice and transgenic mice expressing a non-membrane, GPI anchorless form of PrP (tg88). Though hypothesized that EAAT activity in PrP anchorless astrocytes would be the same as PrP non-expressing astrocytes, tg88 EAAT activity did not mimic PrP KO astrocytic EAAT activity. Further co-immunoprecipitation studies did not reveal a strong interaction between WT PrP and EAAT1, the transporter subtype responsible for the majority of transport activity in primary astrocytes. Thus, in these models, detectable evidence for PrP and EAAT interaction within the lipid raft was not found.

INTRODUTION

Increased EAAT activity in PrP KO astrocytes and the consequent ability to reduce neuronal death following exposure to toxic levels of L-glutamate suggested that expression of PrP regulated L-glutamate transport (Chapter 2). This modulation of transport could be a result of upstream signaling events including RNA and protein expression, unique to PrP expression. However, the association of PrP and the EAATs to the plasma membrane may provide a location where these molecules can interact

PrP is a cell surface glycoprotein, bound to the outer leaflet of the plasma membrane, attached by a GPI anchor (Stahl et al., 1987). GPI linked proteins are typically localized to lipid rafts. In agreement, processed PrP is found in detergent insoluble, cholesterol rich lipid raft microdomains (Naslavsky et al., 1997). However, the physiological relevance of PrP localization to lipid rafts is unknown except in susceptibility to TSE diseases (Speare et al., 2010). Attachment to the plasma membrane is unique in amyloid diseases; TSEs are the only diseases in which the converted protein is GPI anchored. However, transgenic mice that express PrP without the GPI anchor (tg44) appear normal and are still susceptible to TSEs although they present with markedly different clinical symptoms, pathology and incubation period (Chesebro et al., 2005; Chesebro et al., 2010). Presumably, the physiological role of PrP takes advantage of lipid raft locale as either an area in which PrP can modulate the action of other proteins or, itself, be modulated.

The EAATs are trimeric, membrane-spanning proteins responsible for buffering and transporting L- glutamate from the synaptic space. Astrocytic (EAAT1, EAAT2) and neuronal (EAAT3, EAAT4) L-glutamate transporters are localized to cholesterol rich, detergent insoluble, lipid rafts (Butchbach et al., 2004). Localization of neuronal L-glutamate transporters to lipid rafts influences the efficacy of L-glutamate transport through the clustering of proteins that can modulate transport. Interaction of GTRAP3–18 and EAAT3 has been shown to negatively modulate L-glutamate transport (Lin et al., 2001). In contrast, GTRAP41 and GTRAP48 have been shown to increase EAAT4 mediated L-glutamate transport (Jackson et al., 2001). To date, lipid raft proteins which interact with astrocytic EAATs have not been identified.

In order to understand whether PrP negatively modulates astrocytic EAAT activity, the interaction between PrP and EAATs were investigated. Co-immunoprecipitation experiments were performed investigating whether a strong interaction between PrP and the EAATs existed. EAAT activity was also investigated comparing astrocytes harvested from mice expressing GPI anchorless PrP (tg88), membrane anchored, wild-type PrP (WT) or no PrP at all (PrP KO).

METHODS

Animals

C57BL/10SnJ (WT) mice were purchased from Jackson Labs (Bar Harbor, ME). Homozygous PrP knockout (PrP KO) mice on the 129/Ola background (Manson et al., 1994) were backcrossed nine times to C57BL/10SnJ mice selecting for the PrP KO allele by PCR identification at each cross (Race et al., 2009). Transgenic GPI anchorless PrP mice (tg44+/−) (Chesebro et al., 2005) were backcrossed to C57BL/10SnJ-Prnp−/− mice for six to nine generations with selection for the Prnp−/− genotype and the tg44+/− genotype. Heterozygous tg44+/- were then interbred to create homozygous transgenic GPI anchorless PrP mice (tg44-/-, also known as tg88). Thus these mice expressed anchorless PrP but lacked normal anchored mouse PrP allele (Chesebro et al., 2010).

Astrocyte 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% CO_2 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). Purified astrocyte cultures were harvested with trypsin. For D-aspartate uptake assays, astrocytes were reseeded at 1 x 10^5 cells/well in 12 well plates and maintained with fresh media every three days. In some experiments, astrocytes were assayed seven days post-seeding, upon reaching confluence. In co-immunoprecipitation experiments,

confluent 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.

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 KH_2PO_4, 0.3 mM Na_2HPO_4, 1.1 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM HEPES, pH 7.4) and pre-incubated at 37 °C for 5 min. Transport rates were determined using ^{3}H-D-aspartate, which is effectively transported as an EAAT substrate, yet not metabolized by cells following uptake (Koch et al., 1999).Transport assays were carried out in which uptake was initiated by replacing the preincubation buffer with buffer containing ^{3}H-D-aspartate (5-300μM, 2-12μCi/ml). Following a five-minute incubation, 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 $\pm$ 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 (Graph Pad Prism 5, GraphPad Software Inc, La Jolla, CA).

Co-immunoprecipitation

Purified WT astrocyte cultures were washed briefly in 5ml cold PBS. Astrocytes were lysed by shaking incubation with 1ml cold lysis buffer (5mM Tris HCl p.H 7.4, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 1X Protease Inhibitor Cocktail) at 4°C for 5 minutes. Lysates were placed in a pre-cooled 1.5ml centrifuge tube and centrifuged at 100*g* for 15 minutes. Supernatants were collected and stored at -20°C. Protein concentration was quantified using the bicinchoninic acid method (Pierce, Rockford,IL).

Immunoprecipitation of PrP was done by incubating 0.1mg astrocyte lysate with 0.1M NaCl, antibodies specific for PrP: R30, R20, or R18 at indicated concentrations

and co-IP buffer (50mM Tris HCl pH 7.5, 15mM EDTA, 100mM NaCl and 0.1% Triton X-100 and 1X Halt Protease Inhibitor) to a final volume of 500µL. Antibody-lysate complexes were incubated on ice for 90 minutes and occasionally inverted. A Protein A-sepharose slurry was added to the tubes (50µL) and incubated with rotation for 45 minutes at 4°C. Immunoprecipitates were pelleted by centrifugation (1000rpm, 30 seconds) and washed 3 times with 1 ml co-IP buffer. Immunoprecipitates were collected by boiling pellets for 5 minutes, centrifugation to pellet the beads, and collection of the supernatant.

Immunoblot

One half of immunoprecipitate eluate or cell lysates (10ug) were boiled in 1X Sample Buffer containing Reducing Agent (Invitrogen, Carlsbad, CA) for 3 minutes and loaded onto 4-12% Bis Tris gels (for EAAT1) and 12% Tris-Glycine gels (for EAAT2). 4-12% Bis-Tris gels, used for assessing monomeric and multimeric EAAT1 that runs at 64kDa and above, were run at 120 V for 4 hours in MOPS buffer. 12% Tris Glycine gels that were used to assess PrP expression, which runs at 33 KDa, were run for 1 hour at 120V. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane (37V, O/N). Membranes were blocked in TBS with 0.1% tween 20 containing 5% milk. Membranes were probed with either antibodies specific for EAAT1 (1:2000, Tocris Cookson, St. Louis MO), PrP (1:5000, D13, Williamson et al. 1998) or actin (1:10,000, Sigma Aldrich, St. Louis, MO) in TBS–tween for one hour at room temperature. Following three ten-minute washes with TBS-tween, membranes were probed with appropriate horseradish peroxidase conjugated secondary antibodies (EAAT 1: goat anti rabbit (1:3000); PrP: rabbit anti human (1:3000); actin: goat anti-mouse (1:3000)) diluted in TBS tween for one hour at room temperature. Visualization was performed using chemiluminescence, according to manufacturer's recommendations (GE Healthcare Life Sciences, Pittsburg, PA).

RESULTS

Immunoprecipitation of PrP does not bring down EAAT1

Though PrP KO astrocytes exhibited higher EAAT activity compared to WT astrocytes, detectable differences in mRNA expression were not observed, as described in Chapter Two. In contrast to transport differences being mediated by expression level, it is possible that PrP interacts with the EAATs, decreasing the rate transport. Therefore, a direct interaction between PrP and EAAT1, the dominant transporter operational in this primary culture system, was investigated.

PrP was immunoprecipitated from WT astrocyte cultures and protein-protein interactions with EAAT1 were investigated. Low amounts of PrP were immunoprecipitated when astrocytes were lysed in a non-ionic detergent buffer (Figure 1). Therefore, astrocytes were lysed in 0.5% sodium deoxycholate, 0.5% Triton X-100 and incubated alone or in combinations of three polyclonal rabbit antibodies specific for PrP – R30 (89-103), R18 (142-155), and R20 (218-232). R30 alone was most efficient at immunoprecipitating PrP (Figure 2). The addition of other antibodies in conjunction with R30 did not increase the amount of protein immunoprecipitated. Monomeric EAAT1, which in primary astrocyte cultures runs at approximately 65 kDa was not observed in lysates that had been immunoprecipitated. EAAT 1 was also not co-immunoprecipitated in primary astrocytes treated with dbcAMP (Figure 3). The absence of EAAT1 co-immunoprecipitates suggested that if any interaction between PrP and EAAT1 existed, it was not preserved under these lysis conditions.

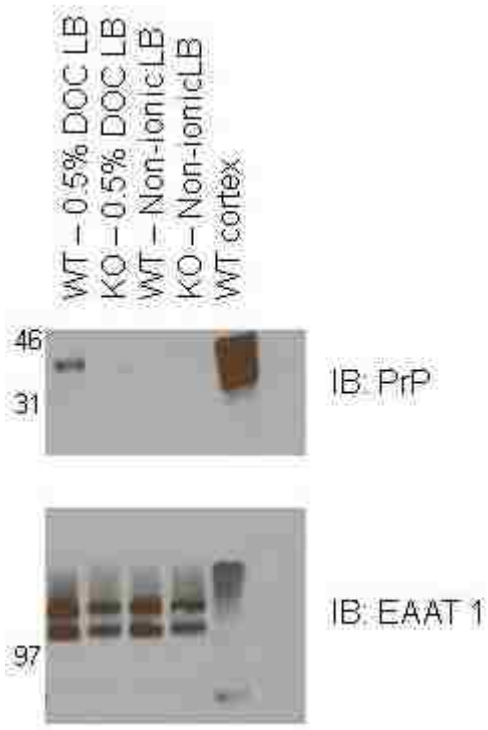

Figure 3-1.

Assessment of ionic versus non-ionic detergent containing lysis buffers.

Two different lysis buffers were investigated to determine which was most effective at solubilizing PrP and EAAT1. Astrocyte cultures lysed in a nonionic detergent consisting of 1% Triton-X100 was able to solubilize EAAT1 to the same degree as the ionic detergents consisting of either 0.5% sodium deoxycholate or 1% sodium deoxycholate, although interestingly, only multimeric forms of EAAT1 was detected. In contrast, a marginal amount of PrP was solubilized with the non-ionic detergent compared to the amount of PrP solubilized with 0.5% sodium deoxycholate. Therefore, in co-immunoprecipitation experiments, 0.5% sodium deoxycholate was used as the astrocyte lysis buffer.

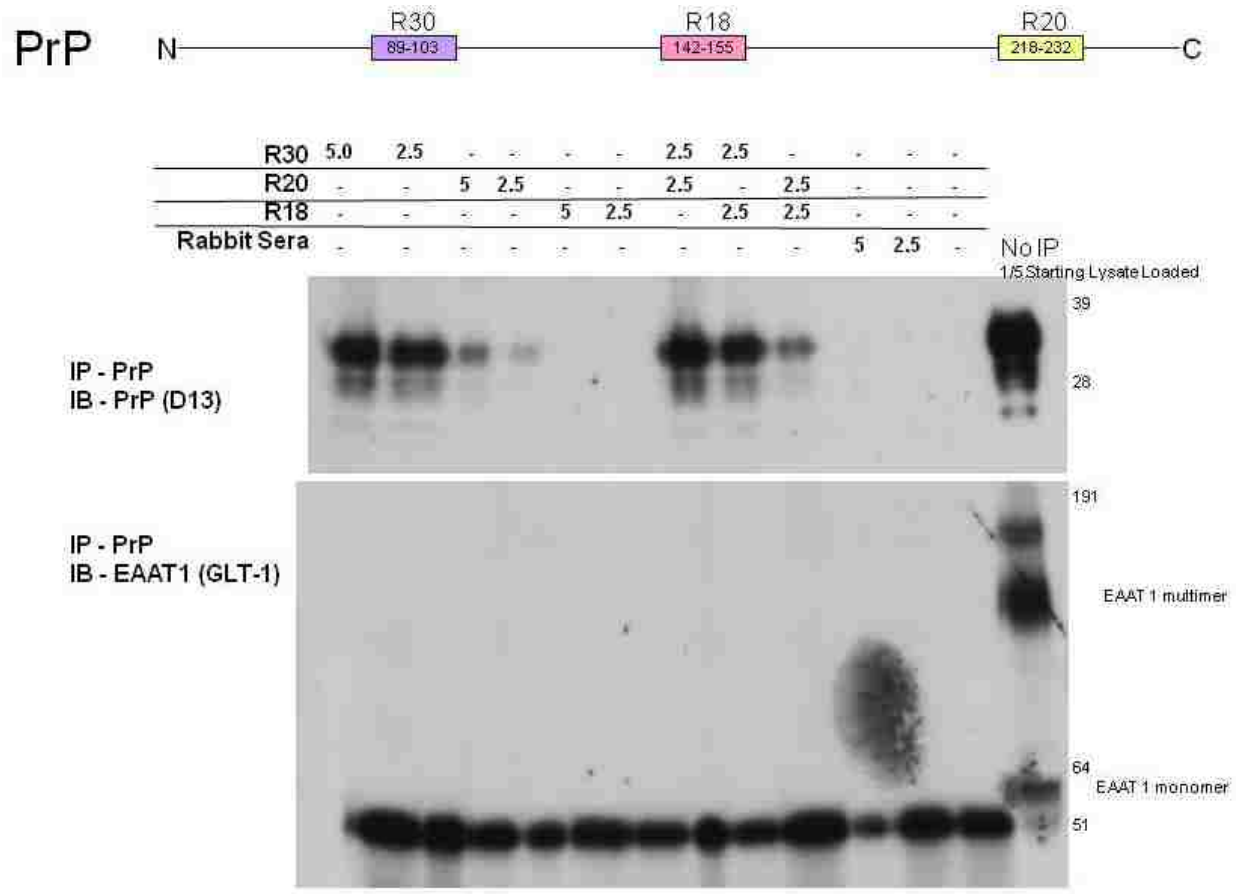

Figure 3-2.

Co-immunoprecipitation of PrP and EAAT1 in primary WT and PrP KO astrocytes.

Astrocyte lysates were immunoprecipitated with 5ul and 2.5ul R30, R20 or R18. Lysates were also immunoprecipitated with 2.5ul each of two of the above antibodies in combination to see if more PrP could be latticed together and pulled down. 5ul and 2.5ul R30 pulled out the greatest amount of PrP from WT astrocyte lysates compared to R20 and R18 alone. Compared to R30 alone, a detectable increase in the amount of PrP pulled out with antibody combinations containing R30 was not observed. Interestingly, the combination of R20 and R18 pulled out more PrP than either R20 or R18 alone. Therefore, R30 was the best antibody to use for immunoprecipitating PrP. However, EAAT1 was not detected in any of the immunoprecipitates. The band running approximately 50 KDa corresponds to IgG heavy chain.

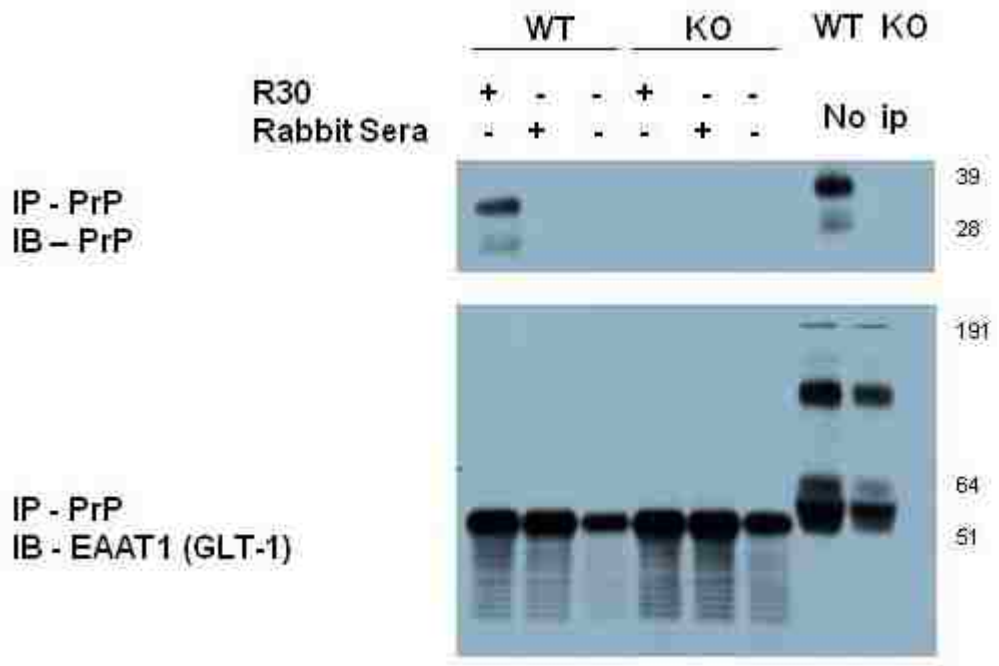

Figure 3-3.

Co-immunoprecipitation of PrP and EAAT1 in primary WT and PrP KO astrocytes treated with dbcAMP.

Astrocyte lysates were immunoprecipitated with 2.5ul R30 and immunoblotted against PrP and EAAT1. EAAT1 was not detected in immunoprecipitated lysates. The band running at approximately 50kDa corresponds to IgG heavy chain.

Astrocytes expressing anchorless PrP do not differ in D-Aspartate transport

Lipid raft microdomains provide a membrane environment conducive to the clustering and interaction of membrane bound proteins. Tg88 transgenic mice express anchorless PrP that lacks the glycophosphatidylinositol anchor and thus is no longer associated with the membrane and lipid raft, but is secreted (Chesebro et al., 2005). Therefore, if similar location of PrP and the EAATs affected L-glutamate transport rates, the absence of PrP from the membrane in Tg88 astrocytes should increase the EAAT activity. Astrocytes from tg88 neonates were cultured and Na+ dependent transport of D-aspartate was measured. At higher concentrations of substrate, tg88 astrocytes did not significantly transport D-aspartate differently than WT or PrP KO astrocytes. The V_{max} of transport in tg88 astrocytes was also not significantly different from WT or PrP KO astrocytes (tg88: 487$\pm$ 32, WT: 396$\pm$31, PrP KO: 657$\pm$44 pmol/min/mg). Though

tg88 mice lacked PrP expression on the cell membrane, their transport of substrate did not mimic transport observed in KO astrocytes.

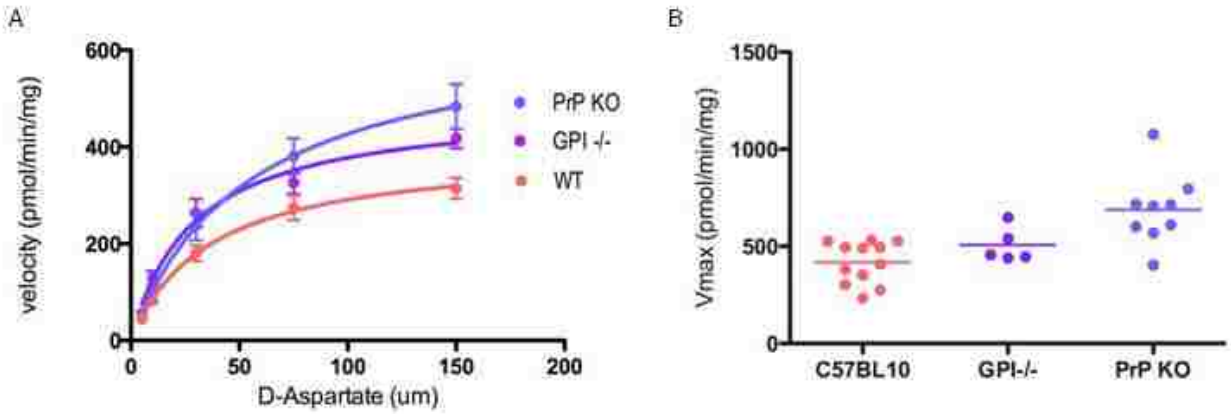

Figure 3-4.

Comparison of D-aspartate transport by EAATs in WT, PrP KO and GPI-/- astrocytes.

A. EAAT activity was measured in primary astrocytes derived from WT and PrP KO neonatal mice cultured 7 days post-harvest. Astrocytes were incubated with 5uM, 10uM, 30uM, 75uM or 150uM D-aspartate, a non-metabolized analog of L-glutamate, for 5 minutes. Transport rate at each concentration was measured and then fit to the Michaelis Menten equation using non-linear regression. Results are averages of data from multiple independent observations. Results show faster transport by PrP KO astrocytes.

B. The Vmax of EAAT-mediated transport for each experiment examining primary astrocytes 7 days post harvest is shown. Results show intermediate Vmax values in GPI-/- astrocytes compared to WT or PrP KO astrocytes (tg88: 487$\pm$ 32, WT: 396$\pm$31, PrP KO: 657$\pm$44 pmol/min/mg).

DISCUSSION

Because of the increased EAAT1 activity observed in PrP KO astrocytes, we hypothesized that homeostatic levels of EAAT activity are governed by a direct interaction between PrP and EAAT1. Both PrP and EAAT1 are membrane-associated proteins localized to lipid rafts, a site where they could interact with each other either directly or indirectly. For example, the unstructured N-terminal domain of PrP (residues 22-90) could transiently occlude the ability of glutamate to enter the bowl-shaped EAAT and reduce its transport into the astrocyte.

However, co-immunoprecipitation experiments failed to reveal a strong protein-protein interaction between PrP and EAAT1 although the experimental approach may have prevented an ability to see such an interaction. The lysis conditions used to extract

PrP contained an ionic detergent, which may have broken apart a protein-protein interaction. It is possible that an interaction between PrP and EAAT1 was weak and would not be maintained by the co-immunoprecipitation procedure, as might be expected with a transient interaction. Because of previous data based on mRNA expression and pharmacology of the PrP KO and WT astrocyte cultures, the majority of transport was determined to be by the EAAT1 subtype (Chapter 2). Thus, co-immunoprecipitation experiments specifically looked for an interaction of PrP with EAAT1 and not with EAAT2, which is expressed and functional albeit at markedly lower levels compared with EAAT1. The possibility exists that modulation of glutamate transport by PrP is subtype specific and that it acts on the less expressed EAAT2.

It is also possible that a direct interaction between PrP and the EAATs is not what modulates transporter efficiency but location does. EAAT1, EAAT2 and PrP are all localized to lipid rafts and such an environment may be conducive to the clustering and interaction of proteins that serve to regulate levels of EAAT activity. If location was indeed what mitigated transport,it was hypothesized that the lack of PrP membrane anchoring in tg88 astrocytes would cause transport activity to mimic PrP KO astrocytes.. However, in tg88 astrocytes, moderate levels of transport were observed, not different from WT or PrP KO astrocytes to which they were compared. However, expression levels may explain why tg88 astrocytes demonstrate a phenotype similar to both PrP KO and WT astrocytes. Tg88 astrocytes express a secreted PrP but at levels 4-5 times less than WT PrP. If a direct interaction between PrP and EAAT1 did occur, possibly it could happen between secreted PrP and EAAT1 and thus, the effect of PrP on EAAT activity could be PrP dose dependent.

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CHAPTER FOUR

EVALUATION OF EXCITATORY AMINO ACID TRANSPORTER ACTIVITY IN PRION PROTEIN EXPRESSING CORTICAL ASTROCYTES

By

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ABSTRACT

Though highly expressed in the central nervous system, the function of prion protein (PrP) is unknown. Abnormalities observed in PrP knockout (PrP KO) mice suggest a function in L-glutamate mediated neurotransmission. Previously, we showed that transport of D-aspartate, a non-metabolized L-glutamate analog, through excitatory amino acid transporters (EAATs) was faster in astrocytes from PrP knockout (PrP KO) mice than in astrocytes from C57BL/10SnJ wild-type (WT) mice. However, since PrP KO mice contained 98.5% sequence identity to the WT strain they were compared to, the difference in EAAT activity could be attributed to the influence of allelic variants in other genes. Therefore, evaluation of EAAT activity in astrocytes harvested from transgenic PrP expressing mice with mixed genetic backgrounds was performed. Astrocytes harvested from a PrP over-expressing mice (tga20 +/-) did not transport D-aspartate faster than PrP null littermates (tga20 -/-). Astrocytes harvested from mice expressing hamster PrP only on neurons (tgNSE) did not transport D-aspartate faster than hamster PrP over- expressing astrocytes (tg7). Experiments altering PrP expression using PrP silencing in WT astrocytes and PrP transduction in PrP KO astrocytes had varied results due to variable expression changes. Taken together, these studies suggest WT expression levels of PrP may regulate EAAT activity.

INTRODUCTION

Though evolutionarily conserved, developmentally regulated, and highly expressed in numerous tissues, the physiological function of prion protein (PrP) is unknown. Mice devoid of PrP expression (PrP KO) develop normally and do not exhibit gross abnormalities (Bueler et al., 1992; Manson et al., 1994). However, PrP KO mice exhibit several subtle abnormalities suggesting that PrP plays a role in cellular homeostasis (reviewed in Chapter one), including maintenance of L-glutamate concentrations in the synaptic cleft through regulating the activity of excitatory amino acid transporters (EAATs) (Chapter Two).

PrP KO mice were originally generated through disruption of the Prnp gene on chromosome 2 by homologous recombination of embryonic stem (ES) cells (Bueler et al., 1992; Manson et al., 1994). In the Edinburgh line of PrP KO mice, ES cells from 129/Ola mice were electroporated with recombinant DNA containing a neo insertion in the Prnp gene (Manson et al., 1994). Following selection, resulting mice were PrP null on a 129/Ola background. Functions of PrP could be assessed comparing these mice to 129/Ola wild type mice and such studies demonstrated that PrP KO mice had decreased LTP (Manson et al., 1995) and mitochondrial abnormalities (Miele et al., 2002).

However, maintenance of the PrP KO line on a 129/Ola background has been notoriously difficult and in many laboratories, mice were crossed to better breeding lines of mice such as C57BL/10. Despite multiple backcrosses, genetic purity of the C57BL/10 strain cannot be re-established and genes immediately flanking the Prnp gene locus will be of 129/Ola origin. The PrP KO mice used to assess the influence of PrP on EAAT activity were on the C57BL/10 background though areas immediately surrounding the Prnp gene locus may have 129/Ola genetic determinants (Figure 4-1). Thus, allelic variation in other genes may have accounted for the differences observed.

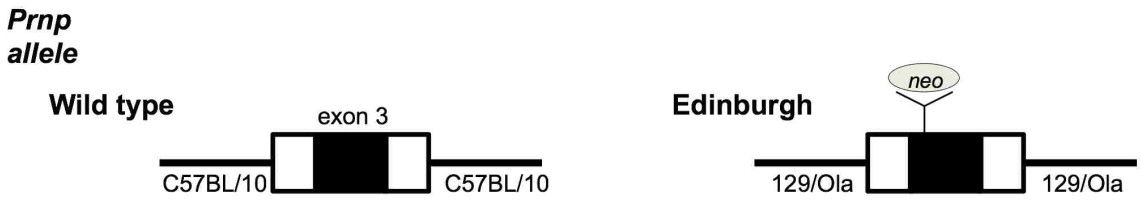

Figure 4-1. Schematic of the Prnp gene on chromosome 2 and the area immediately surrounding the Prnp gene in wildtype and PrP KO mice.

In an effort to distinguish whether abnormal levels of EAAT activity observed in PrP KO astrocytes was due to the lack of PrP expression or flanking genes from the 129/Ola strain, activity was measured in PrP expressing and non-PrP expressing astrocytes. In some experiments, astrocytes were harvested from transgenic lines of mice who contain a neo cassette in the Prnp gene but whose PrP expression is driven on another chromosome. In other experiments, PrP expression was either silenced or induced.

MATERIALS AND METHODS

Animals

C57BL/10SnJ (WT) mice were purchased from Jackson Labs (Bar Harbor, ME). Homozygous PrP knockout (PrP KO) mice on the 129/Ola background (Manson et al., 1994) were backcrossed nine times to C57BL/10SnJ mice selecting for the PrP KO allele by PCR identification at each cross (Race et al., 2009). Homozygous tgNSE (Race et al., 1995), tgGFAP (Raeber et al., 1997) and tg7 (Race et al., 1995) mice, originally on a mixed background were subsequently backcrossed to PrP KO mice to eliminate mouse PrP gene expression, as described (Race et al., 2000). Tga 20 mice (Fischer et al., 1996), on a mixed background, were in the process of being backcrossed to PrP KO mice. Tga20 mice were bred with PrP KO mice and resulting offspring were distinguished by PCR as either heterozygous for the tga20 transgene (+/-) or homozygous null mice for the tga 20 transgene (-/-). This generation of experimental mice was approximately 93% C57BL/10.

Astrocyte 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% CO_2 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). Purified astrocyte cultures were harvested with trypsin. For D-aspartate uptake assays, astrocytes were reseeded at 1 x 10^5 cells/well in 12 well plates and maintained with fresh media every three days. In some experiments, astrocytes were assayed seven days post-seeding, upon reaching confluence.

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 KH_2PO_4, 0.3 mM Na_2HPO_4, 1.1 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM HEPES, pH 7.4) and pre-incubated at 37 °C for 5 min. Transport rates were determined using ^{3}H-D-aspartate, which is effectively transported as an EAAT substrate, yet not metabolized by cells following uptake (Koch et al., 1999). Transport assays were carried out in which uptake was initiated by replacing the preincubation buffer with buffer containing ^{3}H-D-aspartate (150µM, 10µCi/ml). Following a five-minute incubation, 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 (Graph Pad Prism 5, GraphPad Software Inc, La Jolla, CA).

Immunoblot

Purified astrocyte cultures were washed briefly in 5ml cold PBS. Astrocytes were lysed by shaking incubation with 1ml cold lysis buffer (5mM Tris HCl p.H 7.4, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 1X Protease Inhibitor Cocktail) at 4°C for 5 minutes. Lysates were placed in pre-cooled 1.5ml centrifuge tubes and centrifuged at 100*g* for 15 minutes. Supernatants were collected and stored at -20°C. Protein concentration was quantified using the bicinchoninic acid method (Pierce, Rockford, IL). Cell lysates were boiled in 1X Sample Buffer containing Reducing Agent (Invitrogen, Carlsbad, CA) for 3 minutes and loaded 16% Tris Glycine gels that were used to assess PrP expression, which runs at approximately 33-37 KDa . Gels were run for 1 hour at 120V. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane (37V, O/N). Membranes were blocked in TBS with 0.1% tween 20 containing 5% milk. Membranes were probed with antibody specific for PrP (D13, 1:5000 (Williamson et al., 1998)) in TBS –tween for one hour at room temperature. Following three ten-minute washes with TBS-tween, membranes were probed with rabbit anti human (1:3000) diluted in TBS tween for one hour at room temperature. Visualization was performed using chemiluminescence, according to manufacturer's recommendations (GE Healthcare Life Sciences, Pittsburg, PA).

Transfection of primary astrocytes with siRNA

Purified WT and PrP KO astrocytes were plated in 12 well plates at a density of 75,000 cells/well. Approximately four days later, when cell appeared 75% confluent, astrocytes were transfected with 2.5nM, 5nM, 10nM siRNA against Prnp (siRNA ID # s72188, Applied Biosystems, Carlsbad, CA) or 30nM scrambled siRNA or 30nM GAPDH siRNA both of which came with the transfection reagent and were used at concentrations recommended by the manufacturer. siPORT Amine transfection reagent (Applied Biosystems, Carlsbad, CA) was used at 1.5ul per well which was previously determined to be a reagent and concentration sufficient to silence GAPDH expression without causing visible toxicity to astrocytes. siPORT Amine was diluted in 23.5ul

OPTIMEM (Invitrogen, Carlsbad, CA) and incubated for ten minutes at room temperature. Meanwhile, siRNA at the indicated concentrations above was diluted in OPTIMEM to a final volume of 25ul. Both diluted siRNA and diluted transfection reagent were mixed and incubated together at room temperature for an additional ten minutes. Complexes were placed onto cells and incubated for 72 hours in a 37°C, 5% CO2 incubator for 72 hours at which point cells were stained harvested for FACS analysis or assayed for Na+ dependent D-aspartate transport assays. For immunofluorescent staining, astrocytes were plated onto chamber slides and transfection was scaled down to 0.3ul siPORT amine transfection reagent in 10ul OPTIMEM and 10ul diluted siRNA.

Viral transduction of primary astrocytes

PrP KO astrocytes were transduced with supernatant from Psi2 cells who had been tranfected with retrovirus pSFF or pSFF in which hamster PrP had been cloned (graciously given by Suzette Priola, Rocky Mountain Labs, Hamilton MT). Astrocytes were transduced with 500ul stock virus in the presence of 4ug/ml polybrene. In some wells, astrocytes were treated with 4ug polybrene alone. Astrocytes were incubated at 37°C at 5% CO2. Media was completely replaced 24 hours after transduction and assessment of PrP expression by FACS analysis and D-aspartate transport assays performed 7 days after transduction.

Surface PrP staining and FACS analysis

Astrocytes plated in chamber slides were rinsed with PBS 3 times and stained live for PrP using 1ug monoclonal antibody D13, specific for PrP diluted in PBS with 2% FBS. Following 30 minute incubation at 37°C and 3 washes with PBS, astrocytes were fixed in 3.7% formaldehyde for 20 minutes at room temperature, washed and permbealized in 0.1% tx-100 and 0.1% sodium citrate for 10 minutes at room temperature. Cells were then blocked in 0.1M glycine for 30 minutes and with NGS/BSA for an additional 30 minutes. Cells were labeled with anti-GFAP (1:1500) (Dako, Carpinteria, CA) conjugated with AlexaFluor568 goat anti-rabbit IgG (1:3000) (Invitrogen, Carlsbad, CA) to identify astrocytes and AlexaFluor488 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.

For FACS analysis, astrocytes were rinsed with PBS and removed from T-25 flasks by incubation with 0.5mM EDTA for 15 minutes at 37°C. PrP staining was performed on 200,000 live primary astrocytes using 1 μg monoclonal antibody D13, specific for PrP. Following 1 hour incubation at 37°C, cells were fixed in 3.7% formaldehyde for 20 minutes, blocked in 0.1M glycine for 30 minutes and incubated for 1 hour at 22°C with 1 μg AlexaFluor488 goat anti-human IgG (Invitrogen, Carlsbad, CA). Sister tubes of WT and KO astrocytes were fixed, blocked and incubated with AlexaFluor488 goat anti-human IgG alone. Data was collected by FACSCanto II flow cytometer (Becton Dickinson, San Jose CA) and analyzed using FlowJo (Tree Star, Ashland, OR).

RESULTS

PrP KO mice contain non-C57BL10 allelic variants

Though the PrP KO mice used in the experiments described in chapters two and three had been serially backcrossed nine times to homozygous C57BL10 mice, it was possible that genes immediately adjacent to the Prnp gene locus could still be of 129/Ola origin. These genes, which may have differed from the WT mice used in experiments, could have also played a role in the increased transport observed in PrP KO mice. Therefore, DNA from C57BL10 and PrP KO mice were compared using SNP analysis. Non-C57BL10 SNPs were found in submitted PrP KO mice only on chromosome two in a 48 mega base pair region surrounding the Prnp gene locus. This region of differences contains 556 genes, including Slc1a2 which encodes EAAT2.

EAAT activity in astrocytes harvested from transgenic mice expressing hamster PrP

Since genes other than PrP may have contributed to the differences in EAAT activity observed between astrocytes harvested from congenic PrP KO and WT mice, rescue experiments were performed using mice containing a PrP transgene. These transgenic mice were backcrossed to PrP KO mice several times. Importantly, these mice contained the neo insertion in the Prnp gene on chromosome 2 and contained the same flanking genes surrounding the Prnp gene locus (Figure 4-2).

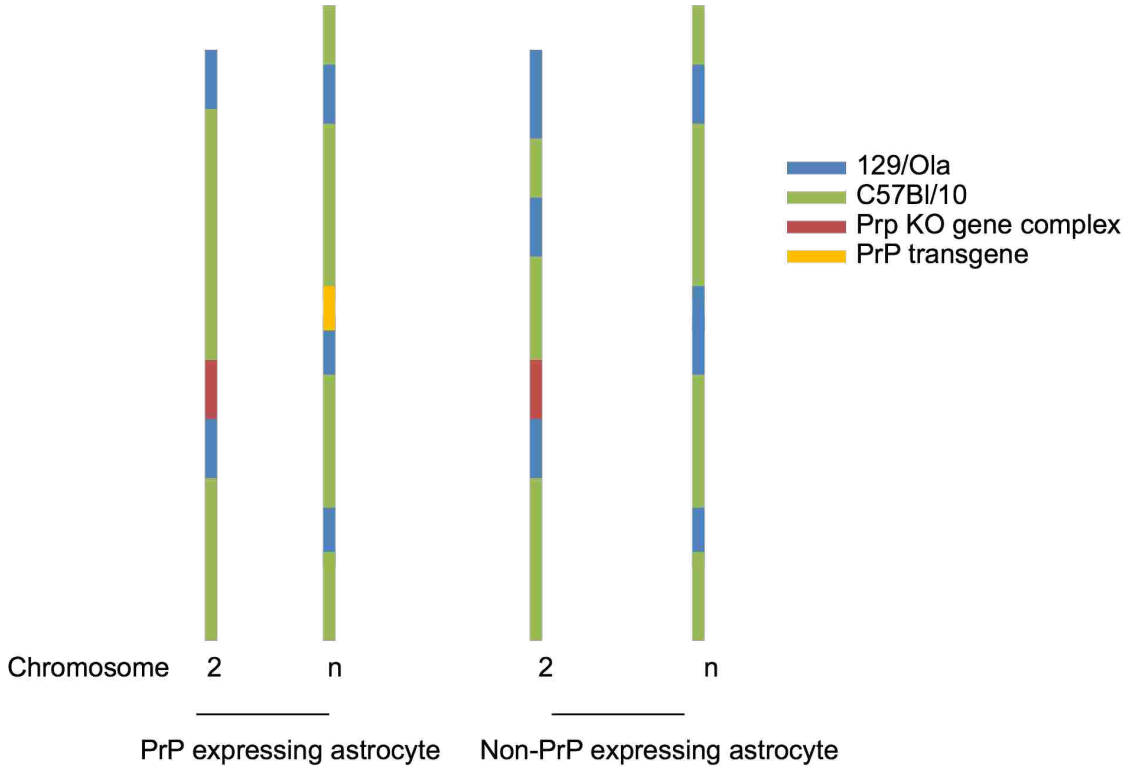

Figure 4-2. Comparison of astrocytes harvested from mice containing a PrP transgene. Transgenic PrP expressing mice contain a neo insertion in the Prnp gene on chromosome 2 as well as genes linked to the Prnp gene locus (red). Transgene insertion on chromosome n leads to cell-specific PrP expression, ie astrocyte specific or neuron specific (yellow). Trangenic mice contain 129/Ola genes (blue) but contain 93-98% genetic identity to the C57BL/10 strain. All astrocytes compared for EAAT activity contain the PrP KO gene complex.

PrP expression was confirmed on astrocytes harvested from hamster PrP expressing transgenic lines of mice. Tg-NSE mice express hamster PrP driven

by neuron specific promoter, NSE. Tg-7 mice overexpress hamster PrP on multiple cell types. As expected, FACS analysis of surface PrP expression demonstrated that tg7 and C57BL10 WT astrocytes express PrP whereas Tg-NSE astrocytes did not express any PrP (Figure 4-3A). Furthermore, levels of PrP expression were higher in tg7 astrocytes, consistent with overexpression in total brain. Immunoblot of whole astrocyte lysate also revealed overexpression of PrP in tg7 astrocytes compared to C57BL/10 WT astrocytes (Figure 4-3B).

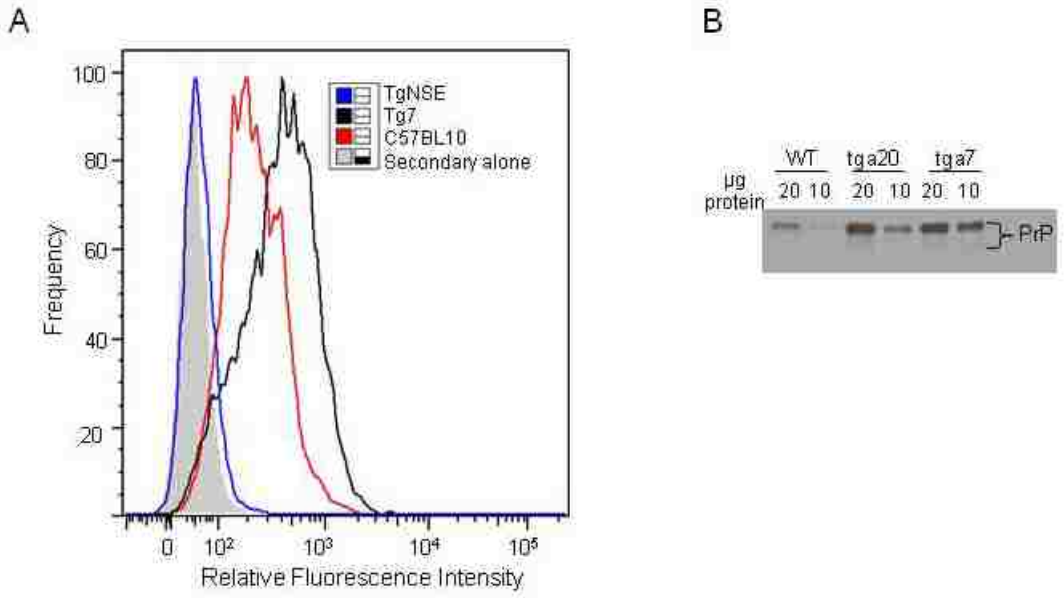

Figure 4-3. PrP expression on primary astrocytes harvested from PrP-expressing transgenic mice.

A. FACS detection of surface PrP expression on astrocytes harvested from C57BL/10, tgNSE and tg7 mice. Live primary astrocytes were labeled with D13, specific for PrP. Only C57BL/10 astrocytes and tg7 astrocytes express PrP. TgNSE astrocytes show the same fluorescent intensity as astrocytes labeled with secondary antibody alone. B. PrP immunoblot of lysates prepared from WT, tga20 and tg7 purified astrocyte cultures.

The influence of PrP expression on EAAT mediated glutamate transport was investigated by measuring EAAT activity in astrocytes harvested from PrP expressing transgenic mice. In these experiments, tgNSE astrocytes, tg7 astrocytes and tgGFAP astrocytes were compared. Tg-GFAP mice express hamster PrP driven by the GFAP promoter, and thus, only express PrP on glial

cells (Raeber et al., 1997) though with expression in total brain much lower compared to WT brains. Sodium dependent transport assays were performed using the non-metabolized EAAT substrate, D-aspartate, at 150uM, a concentration high enough to delineate activity differences (Chapter 2). As shown in Figure 4-4, transport of 150uM D-aspartate was indistinguishable between tgNSE astrocytes, tgGFAP astrocytes and tg7 astrocytes (459$\pm$26, 372$\pm$46, 451$\pm$32 pmol/min/mg respectively, Figure 4-4). Astrocytes were treated with 0.25mM dbcAMP for ten days to increase EAAT1 and EAAT2 expression and thereby increase transport velocities that might distinguish the different transgenic lines of astrocytes (Chapter 2). However, treatment of dbcAMP treatment, while increasing transport, also did not reveal differences between tgNSE, tgGFAP or tg7 astrocytes (597$\pm$42, 658$\pm$34, 588$\pm$94 pmol/min/mg respectively, Figure 4-4). Thus, in both untreated and dbcAMP treated astrocytes, lack of hamster PrP expression did not change EAAT activity compared to low expression of PrP or overexpression of PrP.

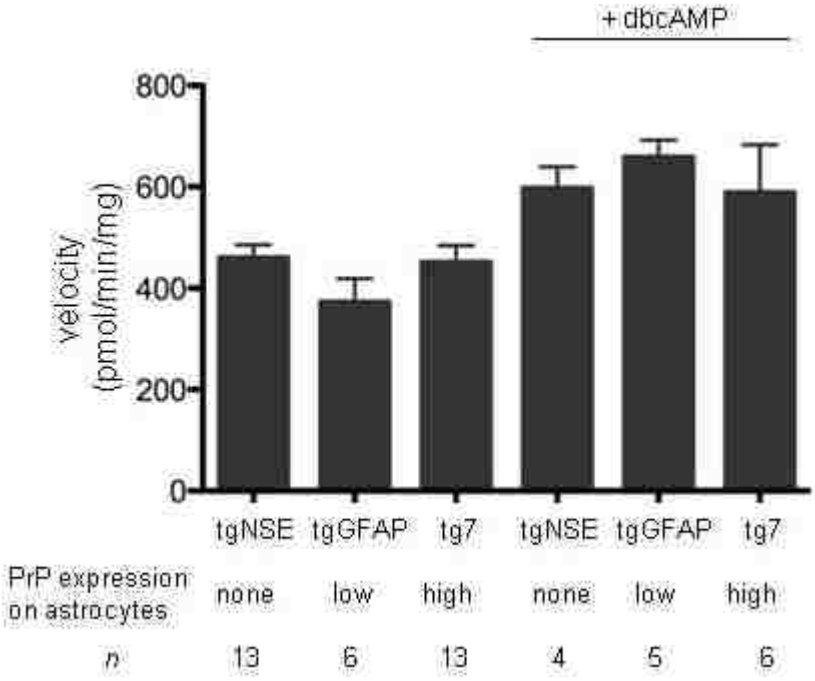

Figure 4-4. EAAT activity in tgNSE, tgGFAP and tg7 astrocyes. Uptake of 150uM D-aspartate was quantified in primary astrocytes cultured from tgNSE, tgGFAP and tg7 mice, which differ in levels of hamster PrP expression.

EAAT activity in astrocytes harvested from transgenic mice expressing mouse PrP

Similar experiments attempting to rescue the difference observed between PrP KO and WT mice were performed on a line of transgenic mice, tga20, who overexpress mouse PrP (Fischer et al., 1996) and were in the process of being backcrossed to PrP KO mice. Littermates were either heterozygous at the tga20 locus and expressed 3-fold higher levels of PrP (tga20 +/-), or were null at the tga 20 locus and did not express PrP (tga20 -/-). Littermates all contained the Prnp null gene complex on chromosome 2 (Figure 4-2). Immunoblot lysates of primary astrocytes harvested from tga20 mice demonstrated overexpression of PrP compared to WT astrocytes (Figure 4-3B). When transport of 150uM D-aspartate was measured in tga20 astrocytes, a difference between tga20 +/- PrP expressing and tga20 -/- PrP non-expressing astrocytes was not observed

(455±40 vs. 483±22 pmol/min/mg respectively, Figure 4-5). A difference in transport velocity was also not observed in dbcAMP treated tga20 +/- vs tga20 -/- astrocytes (947±68 vs. 741±62 pmol/min/mg, respectively, Figure 4-5). Thus, overexpression of mouse PrP compared to non-expression also did not rescue the EAAT activity differences observed between WT and PrP KO astrocytes.

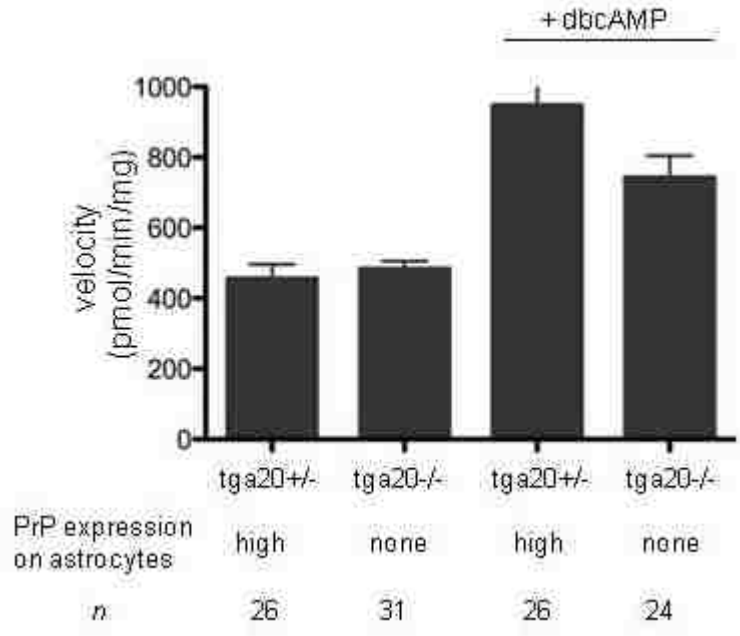

Figure 4-5. EAAT activity in tga20 +- and tga20 -/- astrocytes. Uptake of 150uM D-aspartate was quantified in primary astrocytes cultured from tga20 +/- and tga20 -/ astrocytes which overexpress or do not express mouse PrP.

EAAT activity in KO astrocytes transduced to express PrP or WT astrocytes with silenced PrP expression

Usage of transgenic mice to describe EAAT activity as a function of PrP expression is still prone to the influence of background genes. Therefore, EAAT mediated D-aspartate transport was measured in WT astrocytes whose PrP expression was silenced and PrP KO astrocytes induced to express PrP.

To rescue the PrP KO phenotype, PrP expression was silenced in WT astrocytes by transfecting Prnp specific siRNA, as described in Methods. Levels of PrP silencing between experiments were variable and may have depended on how the detection method of PrP expression. In some experiments when immunoblot and immunostaining of PrP KO astrocytes were employed, silencing

specific to siRNA against Prnp compared to a scambled siRNA sequence was observed (data not shown). However, FACS analysis showed surface reduction of PrP expression independent of the siRNA sequence (Figure 4-6A.). When the effect of silencing PrP on EAAT activity was assayed, an increased in D-aspartate transport was observed in all conditions and was probably the result of siRNA transfection (Figure 4-6B).

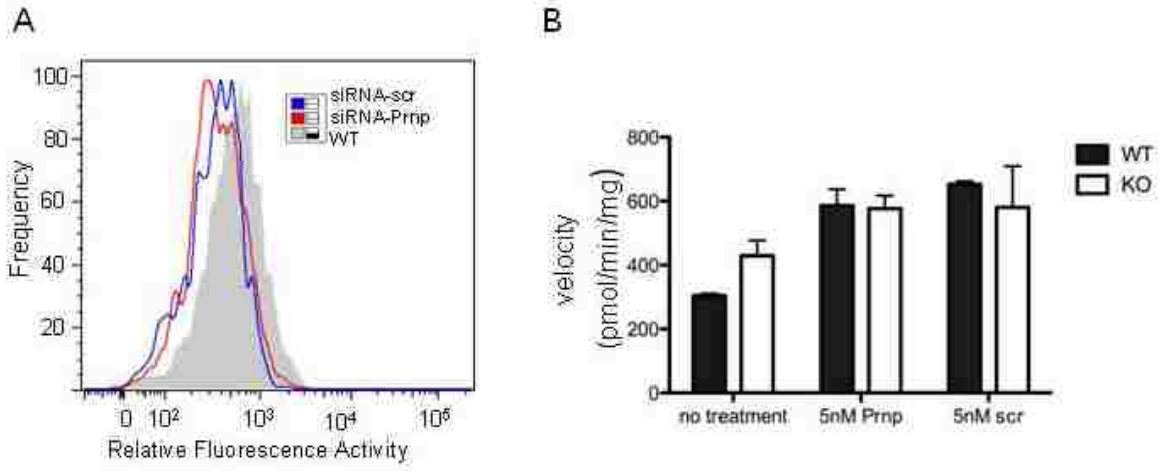

Figure 4-6. Knockdown of PrP expression following transfection with Prnp specific siRNA. A. FACS detection of surface PrP expression on C57BL10 WT astrocytes 72 hours following transfection with siRNA against PrP or a scrambled siRNA sequence. Live primary astrocytes were labeled with D13, specific for PrP. expression. In this experiment, a reduction in PrP expression is observed independent of siRNA sequence. B. Uptake of 150uM D-aspartate was quantified in primary PrP KO and WT astrocytes following 72 hours transfection with either siRNA against PrP or a scrambled sequence. In all treatments, there was an increase in EAAT activity.

To rescue PrP expression in PrP KO astrocytes in an attempt to mimic the WT phenotype, PrP expression was induced in PrP KO astrocytes and EAAT activity was measured. PrP KO astrocytes were transduced in the presence of polybrene with retrovirus pSFF in which hamster PrP had been cloned. While levels of expression varied experiment to experiment (ranging from 45% - 60%, Figure 4-7A), PrP KO astrocytes did express PrP. However, PrP KO astrocytes expressing PrP did not exhibit reduced transport compared to PrP KO astrocytes not expressing PrP (Figure 4-7B).

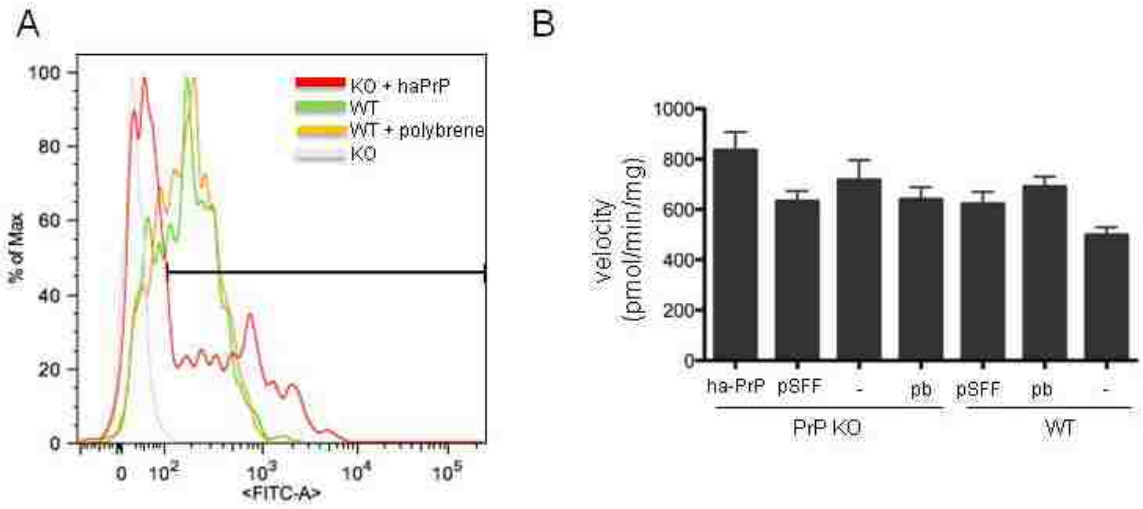

Figure 4-7. EAAT activity following induction of PrP expression in PrP KO astrocytes. A. FACS detection of surface PrP expression 7 days following transduction with pSFF-haPrP in the presence of 4ug/ml polybrene (pb). Live primary astrocytes were labeled with D13, specific for PrP. expression. Compared to PrP KO astrocytes, 50% transduced astrocytes expressed PrP. B. Uptake of 150uM D-aspartate was quantified in PrP KO and WT astrocytes seven days following transduction. PrP KO astrocytes transduced to express PrP did not have decreased transport compared to untreated KO astrocytes.

DISCUSSION

The experiments described in this chapter were aimed at replicating the observation that PrP KO astrocytes have higher EAAT activity than WT astrocytes. The phenotype observed could not be definitively attributed to PrP expression; the mice compared were genetically identical except at chromosome 2 in the region surrounding the Prnp gene. Here, 129/Ola genes existed in PrP KO astrocytes, different than the C57BL10 astrocytes, and thus differences observed could be due to the expression of these 129/Ola genes. The gene encoding EAAT2, Slc1a2, is located in this region and according to SNP analysis, differs between WT and PrP KO mice. Of note, most of the transport in the system studied was due to EAAT1 encoded by Slc1a3, located on a chromosome 11 and lacking any genetic differences between PrP KO and WT mice.

These experiments used astrocytes harvested from transgenic mice whose PrP expression driven by a promoter other than the PrP promoter and

whose genome contained the Prnp KO gene complex. If 129/Ola genes flanking the Prnp KO gene locus contributed to the differences observed in PrP KO versus WT astrocytes, these transgenic mice would have the same 129/Ola genes and EAAT would be similar. In experiments examining EAAT activity in PrP-expressing transgenic mice, PrP expression did not rescue the WT phenotype. The simplest explanation therefore is expression of129/Ola genes in the area surrounding the Prnp KO gene locus did play a role in the EAAT activity differences observed between WT and PrP KO astrocytes.

However, there are important caveats to interpreting EAAT activity in PrP expressing transgenic mice. The tga20 mice used in this study are of a mixed background. Other genes on other chromosomes may be different between the lines of mice compared, i.e. Slc1a3 encoding for EAAT1. Furthermore, the PrP expressing transgenic mice did not express wild-type levels of PrP. Interestingly, the two comparisons that showed similar levels of transport, tgNSE vs tg7 and tga20+/- vs. tga20-/-, both compared the lack of PrP expression to the overexpression of PrP. Transgene expression can lead to phenotypes by itself either due to site of insertion, abnormal expression or overexpression. Tga20 mice have disturbed T cell development (Jouvin-Marche et al., 2006; Zabel et al., 2009), increased susceptibility to kainic acid induced seizures and decreased synaptic facilitiation and long term potentiation (Rangel et al., 2009). Aggregation of PrP has been described in other PrP overexpressing transgenic mice (Westaway et al., 1994; Chiesa et al., 2008). The inability, therefore, of the rescue experiments to mimic EAAT activity observed in PrP KO and WT mice may be due to other reasons unrelated to the Prnp gene and genes flanking the region on chromosome two.

Multiple approaches to understanding the influence of PrP on EAAT activity were taken. This includes assessment of another PrP knockout line, Zurich PrP $^{0/0}$ and the strain used to make the knockout mouse, 129/Ola (data not shown). However, because of the same caveats described, interpretation is limited. With siRNA and viral transduction experiments, the efficiency of manipulation was variable and made comparisons against untreated astrocytes

difficult. The best comparison performed was that comparing C57BL/10 wildtype and PrP KO mice who share 98.6% genetic identity. The only comparison better would be studying the PrP knockout on the 129/Ola background, which will be accomplished in future studies.

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CHAPTER FIVE

CONCLUSION

Two questions hover over the fields of prion protein (PrP) biology and excitatory amino acid transporter (EAAT) biology: what is the function of PrP and how is EAAT activity controlled. Multiple lines of evidence demonstrate that both PrP and the EAATs regulate aspects of L-glutamate neurotransmission though a link between them has not been demonstrated. The overall aim of this dissertation research was to use PrP KO mice as a tool to characterize the influence of PrP on astrocytic EAAT mediated L-glutamate transport. We found that astrocytes harvested from PrP KO mice displayed abnormal EAAT activity that was mediated primarily by the EAAT1 subtype. However, the similar localization of the EAATs and PrP to lipid rafts did not appear to control EAAT activity nor did overexpression of PrP. Thus, WT PrP expression appeared to regulate astrocytic EAAT activity.

How might PrP be regulating EAAT activity? EAAT activity is regulated at multiple levels: DNA transcription, translation, post-translational modifications and localization. At the level of detection, PrP expression did not influence transcriptional regulation of EAAT1 or EAAT2 (Chapter 2). PrP expression may influence post-translational modifications of the EAATs that may subsequently affect functional properties of the EAATs. For example, EAAT 1 is glycosylated in the extracellular loop of transmembrane helices 3 and 4 (Conradt et al., 1995) and it was not addressed in this research whether PrP expression alters the ratio of unglycosylated versus glycosylated forms. However, the role of EAAT glycosylations is unknown as both forms traffic to the plasma membrane and show similar levels of uptake activity (Conradt et al., 1995; Raunser et al., 2005). Therefore, the likelihood that PrP affects glycosylation of the EAATs is possible though mediating functional outcomes is unlikely.

If not through transcriptional regulation or post-translational modification, PrP may influence EAAT activity by location. Both PrP and EAATs are localized to lipid rafts (Stahl et al., 1987; Butchbach et al., 2004) and such a locale may be conducive to a direct or indirect interaction between them. In Chapter 3, we investigated the importance of PrP lipid raft localization to EAAT mediated transport by utilizing PrP mutant mice

(tg88) which express a soluble form of PrP. Uptake measurements in tg88 astrocytes did not demonstrate transport activity that was dependent on lipid raft location. However, it is possible that soluble PrP can still bind extracellularly to the EAATs, therefore never necessitating membrane localization to control transport. EAAT interaction with intracellular proteins in the lipid raft have been identified: GTRAP 41 and GTRAP 48 increases EAAT4 activity (Jackson et al., 2001) while GTRAP 3-18 decreases EAAT 3 activity (Lin et al., 2001). All three EAAT interacting proteins, the only ones identified so far, act on neuronal EAATs through binding to the C-terminal intracellular domains. It is possible that PrP is an astrocytic EAAT interacting protein that decreases EAAT activity through interaction with the extracellular domains on EAAT1 and/or EAAT2.

The EAATs are also subject to regulation through membrane trafficking and it is possible that PrP exerts its influence on EAAT activity by affecting local concentrations of EAAT protein on the membrane. Pre-incubation with L-glutamate has been shown to alter membrane expression of EAAT1, which was prevented by actin disrupting agents (Duan et al., 1999). It has been suggested that EAAT2 can be rapidly internalized by clathrin-mediated endocytosis (Zhou and Sutherland, 2004; Susarla and Robinson, 2008). Alterations in membrane levels of EAAT1/2 may be a mechanism to maintain local glutamate concentrations but would require an extracellular sensor (Chaudhry et al., 1995). As an extracellular protein, PrP could act as a sensor and mediate the internalization of astrocytic L-glutamate transporters in response to L-glutamate concentration. Previous studies have suggested that PrP can act as a stress sensor for hydrogen peroxide (Krebs et al., 2007) and copper (Rachidi et al., 2003). In our studies, EAAT activity was measured by exposing WT and PrP KO astrocytes to varying concentrations of D-aspartate. We observed substrate concentration dependant differences in EAAT activity between PrP KO and WT astrocytes. Interestingly, evidence suggests PrP is also internalized via clathrin-mediated endocytosis, which is unusual for a GPI-anchored protein lacking an intracellular sequence necessary for interaction with endocytic adaptor proteins. It is possible that internalization of PrP requires its interaction with an integral membrane protein that contains a localization

sequence for clathrin-mediated endocytosis. Thus, PrP interaction with EAAT 1 and/or EAAT 2 may serve to maintain a local glutamate concentration at the synapse.

EAAT internalization through clathrin-mediated endocytosis was specifically studied on EAAT 2 and whether a similar action of activity dependent internalization of EAAT1 is unknown. In our studies, the transporter subtype responsible for the majority of transport was EAAT1, which was most likely a result of EAAT1 being highly expressed in our cultures. Despite efforts to increase EAAT2 expression through dbcAMP treatment and co-culture with neurons, levels of EAAT2 expression were typically 10 fold lower than EAAT2 in all cultures. Thus, the contribution of EAAT2 to transport in WT and PrP KO astrocytes was overshadowed by the high contribution of EAAT1. We hypothesized that the influence of PrP on primary astrocyte L-glutamate transport was through modulation of EAAT1 activity and it remains to be seen whether similar modulation by PrP would act on EAAT2 and EAAT3 in the synapse. Future studies looking at transport in synaptosomes, whose pharmacology profile is EAAT2 dominant, will address this issue.

However, if PrP specifically acted on EAAT1 to decrease L-glutamate transport, its contribution to regulating L-glutamate concentrations *in vivo* would not be insignificant. EAAT1 is expressed throughout the brain though, in contrast to primary astrocytes *in vitro*, its expression is lower than EAAT2 whose expression dominates in all areas of the brain except the cerebellum (Danbolt, 2001). The assumption that EAAT2 is the most relevant L-glutamate transporter *in* vivo is most likely due to its high expression. For example, quantitative immunoblotting demonstrated that in the hippocampus, EAAT2 is four times higher than EAAT1 (Lehre and Danbolt, 1998). Over 90% EAAT2 activity is immunoprecipitated from crude forebrain extracts of reconstituted activity (Haugeto et al., 1996) and the pharmacology profile of synaptosomes is mainly EAAT2 (Koch et al., 1999b). Thus, analogous to what we observed in our primary astrocyte cultures, expression differences of EAAT1 and EAAT2 might hide functional contributions to overall L-glutamate transport. Understanding the contribution of other transporters to maintenance of L-glutamate homeostasis is overshadowed by EAAT2 dominant expression. Interestingly, pharmacological inactivation of EAAT2 did not lead to widespread neuronal death as expected, suggesting that other L-glutamate

transporters such as EAAT3 and EAAT 1 can be rapidly mobilized to clear L-glutamate (Selkirk et al., 2005).

The importance of EAAT1 is poorly understood *in vivo*. EAAT1 is the dominant transporter expressed during development in all regions of the brain and its expression remains throughout adulthood. EAAT1 is the primary transporter expressed by Bergman glia in the cerebellum and EAAT1 knockout mice suffer from cerebellar ataxia. Interestingly, dysfunction in EAAT1 is implicated in schizophrenia (Karlsson et al., 2009), a disease in which a hypothesized mechanism is decreased glutamate signaling (Miyamoto et al., 2005). PrP KO mice suffer from abnormalities that may be related to dampened L-glutamatergic neurotransmission including reduced synaptic transmission, long term potentiation and poor behavior in learning and memory tasks (Criado et al., 2005). These observations may be the result of premature clearance of glutamate by overactive astrocytic EAATs.

Does abnormal L-glutamate transport contribute to the synaptic dysfunction observed in transmissible spongiform encephalopathies (TSEs)? TSEs are fatal neurodegenerative diseases. Several mammalian TSE strains exist, including bovine spongiform encephalopathy or “mad cow disease” in cattle, scrapie in sheep and goats, chronic wasting disease in deer and elk and Gerstmann Sträussler Scheinker syndrome, familial Creutzfeldt-Jacob Disease (CJD), fatal familial insomnia, variant CJD, and kuru in humans. Notably, the species barrier usually restricts transmission of TSE diseases from one species to another, but as evident with BSE transmission to humans, is possible. Fortunately, the difficulty in crossing the species barrier as well as increased surveillance in human consumed game and livestock has rendered human transmission low; the incidence of disease is one or two cases per million humans. Nevertheless, the spread of disease within an infected herd and threat of human infection has consequently made an enormous economic impact on the livestock and hunting industries.

TSE strains are unique in the targeted species, incubation time, clinical presentation and tissues affected. However, they share common histopathological characteristics. Disease manifests itself as grey matter spongiform vacuolation, neuronal dropout and gliosis, usually colocalizing with PrP^{res}. PrP^{res} is conformationally

distinct from PrP in that it is β-sheet rich and prone to aggregation. PrP^{res} accumulates at the cell surface in intracellular vesicles or as diffuse extracellular deposits (Caughey et al., 2009). Typically, TSEs are characterized by a long incubation time in which most symptoms, in relation to the incubation period, shortly precede death. Spongiform vacuolation may be an early morphological marker as neurons are not lost yet but vacuoles are formed within them (Moreno and Mallucci, 2010). In mouse models, synaptic pathology represents one of the earliest changes seen in the disease (Clinton et al., 1993; Fuhrmann et al., 2007; Mallucci et al., 2007) and results in reduced synaptic responses and behavioral deficits related to spatial learning (Mallucci et al., 2007). The disease progresses into ataxia and wasting. In humans, clinical symptoms are characterized by cognitive decline, dementia and cerebellar ataxia (Aguzzi et al., 2008).

The causes of synaptic pathology in TSEs are unknown but abnormal EAAT activity could be a contributing factor. The direct and indirect vulnerability of neurons to PrP^{res} induced damage demonstrates that the disease takes advantage of the relationship between neurons and astrocytes. Mice expressing PrP only on neurons are susceptible to TSE infection (Race et al., 1995), suggesting that PrP expression on non-neuronal cells is not necessary for disease. However, neurons secrete soluble factors that affect EAAT expression (Gegelashvili et al., 1997; Swanson et al., 1997; Yang et al., 2009) as well as present PrP^{res} extracellularly which may cause compromised astrocytic EAAT function. Interestingly, mice expressing PrP only on astrocytes are also susceptible to TSE (Raeber et al., 1997), demonstrating that neuronal damage from infection can come indirectly. Astrocytes are one of the earliest sites of PrP^{res} accumulation in the brain (Diedrich et al., 1991) and PrP^{res} toxicity might also cause disturbance in astrocytic EAAT activity, thereby perturbing synaptic L-glutamate homeostasis. These abnormalities could precede the development of neuropathological changes but affect more subtle phenotypes such as altered dendritic spine morphology (Fuhrmann et al., 2007) and depressed synaptic responses (Mallucci et al., 2007). Gliosis in the terminal stages of disease may lead to reversal of glutamate uptake (Koch et al., 1999a) in which the EAATs may act as a site of efflux for cellular L-glutamate. Interestingly, increased EAAT1 expression was observed in microglia of patients with

CJD and FFI who had long survival periods suggesting that, though not sufficient to prevent neurological deterioration, microglial expression of EAAT1 might be neuroprotective. If EAAT dysfunction plays a role in pathology of TSE diseases several questions would have to be addressed, such as the duration of exposure to TSE, whether PrP^{res} accumulation is necessary and whether possible dysfunction is great enough to lead to neuronal damage.

The function of PrP may be multidimensional, converging on a role maintaining synaptic specificity in which activity dependent events are targeted towards appropriate neurons and receptors, limiting spillover, excessive activation or premature signal termination. The importance of EAATs in controlling glutamate signaling is region dependent and thus, PrP's influence may be most critical in areas where astrocytes tightly ensheath nerve terminals. Perhaps in other areas where termination of signal operates through diffusion, PrP affects L-glutamate receptor distribution or activation. The symptoms and pathology of TSE infection may take advantage of the role of PrP in the glutamatergic system starting with a pre-clinical stage characterized by low-level synaptic dysfunction and ending with a clinical stage that may be a result of excitotoxicity and transporter reversal. Through modulation of EAAT activity and glutamate receptor dynamics, the function of PrP may be to maintain an optimal level of L-glutamate in the synaptic cleft during all phases of neuronal signaling.

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