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Glutamate Transporter EAAT2 Expression is Up-Regulated in Reactive Astrocytes in Human Periventricular Leukomalacia

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Abstract

The major neuropathological correlate of cerebral palsy in premature infants is periventricular leukomalacia (PVL), a disorder of the immature cerebral white matter. Cerebral ischemia leading to excitotoxicity is thought to be important in the pathogenesis of this disorder, implying a critical role for glutamate transporters, the major determinants of extracellular glutamate concentration. Previously, we found that EAAT2 expression is limited primarily to premyelinating oligodendrocytes early in development and is rarely observed in astrocytes until >40 weeks. In this study, we analyzed the expression of EAAT2 in cerebral white matter from PVL and control cases. Western blot analysis suggested an up-regulation of EAAT2 in PVL compared with control cases. Single- and double-label immunocytochemistry showed a significantly higher percentage of EAAT2-immunopositive astrocytes in PVL ($51.8\% \pm 5.6\%$) compared with control white matter ($21.4\% \pm 5.6\%$; $P = 0.004$). Macrophages in the necrotic foci in PVL also expressed EAAT2. Premyelinating oligodendrocytes in both PVL and control cases expressed EAAT2, without qualitative difference in expression. The previously unrecognized up-regulation of EAAT2 in reactive astrocytes and its presence in macrophages in PVL reported here may reflect a response to either hypoxic-ischemic injury or inflammation.

Indexing terms

cerebral palsy; reactive astrocytes; inflammation; microglia; oligodendrocytes; prematurity

The major neuropathological correlate of cerebral palsy in the premature infant is periventricular leukomalacia (PVL), a lesion in the developing cerebral white matter characterized by focal periventricular necrosis with axonal damage and macrophages along with diffuse inflammation with reactive astrocytes and activated microglia in the surrounding white matter (Haynes et al., 2003). Oligodendrocytes (OLs) appear to be the primary target of cell injury in this disorder, leading to myelin deficits in long-term survivors. Although the causes of PVL are not completely understood, cerebral ischemia is likely to play an important role (Banker and Larroche, 1962; Volpe, 2001), implicating excitotoxicity, i.e., cell death produced by the toxic accumulation of extracellular glutamate

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and excessive activation of glutamate receptors (Choi, 1988; Lipton and Rosenberg, 1994; Meldrum and Garthwaite, 1990). We postulated that glutamate transporters are involved in the pathogenesis of excitotoxic white matter damage in PVL because they provide the only known mechanism for maintaining extracellular glutamate homeostasis and, paradoxically, in ischemia become a source of pathological extracellular accumulation of glutamate (Mitani and Tanaka, 2003; Pines and Kanner, 1990; Rossi et al., 2000; Szatkowski et al., 1990). The glutamate transporter family has five subtypes, known as EAAT1–5 in humans; EAAT1–3 are known as GLAST (EAAT1), GLT1 (EAAT2), and EAAC1 (EAAT3) in the animals, rat or rabbit, in which they were originally discovered (Arriza et al., 1994, 1997; Fairman et al., 1995; Pines et al., 1992; Storck et al., 1992). EAAT2, the focus of this study, is the major transporter in the brain and represents 1% of total brain protein (Haugeto et al., 1996). The EAAT2/GLT1 murine knockout is a lethal mutation; animals die from intractable epilepsy several weeks after birth and have only about 5% of wild-type levels of glutamate uptake activity in cortical synaptosomes (Tanaka et al., 1997). In a previous study in normative human white matter, we found that EAAT2 expression is limited primarily to OLs early in development and is increased during the period when the premature infant is most vulnerable to PVL. Also, EAAT2 expression was rarely observed in astrocytes until >40 weeks (DeSilva et al., 2007).

In PVL, the sources of excessive levels of glutamate likely include reverse glutamate transport in developing OLs (Deng et al., 2003; DeSilva et al., 2007; Fern and Möller, 2000) and vesicular release of glutamate from unmyelinated axons (Ziskin et al., 2007). In a rat model of hypoxic-ischemic injury, semiquantitative estimation of glutamate levels in OLs and axons (measured by immunoelectron microscopy) showed a significant decline after hypoxic-ischemic injury (Back et al., 2007). Given the critical role of EAAT2 both as a potential source of glutamate and in maintaining glutamate homeostasis, we compared the expression of EAAT2 in developing brains of humans diagnosed with PVL to control white matter. Our strategy was twofold: 1) to determine quantitatively by Western blot analysis whether there are changes in EAAT2 expression between PVL and control cases in developing human cerebral white matter and 2) to determine by single- and double-labeling immunocytochemistry the expression of EAAT2 in different cellular components of PVL, i.e., OLs, reactive astrocytes, and activated microglia/macrophages.

MATERIALS AND METHODS

Clinical database

Seventeen PVL cases and sixteen control cases were examined (Tables 1, 2) for single-label immunocytochemistry using diaminobenzidine (DAB). These formalin-fixed, paraffin-embedded samples were obtained from the archives of the Department of Pathology, Children's Hospital Boston. Among these cases, six PVL cases (case 1, 2, 9, 11, 13, 16) and six age-matched control cases (1, 8, 12, 13, 14, 15) had been fixed in 4% paraformaldehyde at autopsy and were suitable for double-label immunocytochemistry. For Western blot analysis, the parietooccipital regions were removed at autopsy and snap-frozen immediately in blocks at -70°C . Three PVL cases (2, 11, and 14; Table 1A) and 21 control cases (DeSilva et al., 2007) were available for Western blot analysis. By definition, PVL histologically comprises two components: 1) focal necrosis in the periventricular region of the cerebral white matter and 2) reactive gliosis and activated microglia in the surrounding cerebral white matter (Folkerth et al., 2004). The controls in this study were defined as cases with no PVL and only minor or no neuropathological findings, as in our previous studies (Billiards et al., 2006; Haynes et al., 2003). Approval of the appropriate Institutional Review Boards was obtained for all studies. The age of the infant cases is expressed in postconceptional (gestational plus postnatal) weeks. Only samples with a post-mortem interval of less than 24 hours were used.

Antibodies for Western blot analysis and immunocytochemistry

A polyclonal antibody against the N-terminus of GLT1 (anti-nGLT1), which detects both variant forms of GLT1, GLT1a and GLT1b, was generated in New Zealand white rabbits (Research Genetics, Huntsville, AL); the antibody has been previously characterized (Chen et al., 2002, 2004) and was used to detect EAAT2 in human tissue (DeSilva et al., 2007). The anti-nGLT1 antibody was generated based on the published sequence for rat (aa 1–15; GenBank accession No. AF451299), which is identical to the human sequence EAAT2 (NM_004171). The variant forms of EAAT2/GLT1 are different only at their C-termini (Chen et al., 2002) so the anti-nGLT1 antibody will detect both variant forms of EAAT2, EAAT2a and EAAT2b, in human tissue in this study. To identify the cellular localization of EAAT2, fluorescent double-labeling immunocytochemistry was performed with the microglial marker CD68 (1:50; No. CMC321; Cell Marque, Austin, TX), the astrocytic marker glial fibrillary acidic protein (GFAP; 1:10,000; No. SMI-22R; Covance, Berkeley, CA), and the O4 monoclonal antibody produced by hybridoma cells (generously provided by Dr. Stephen Pfeiffer) for precursor OLs. A detailed summary of antibodies used in this study is included in Table 3.

Immunoblot analysis of lysates from human PVL and control brains

Lysates were made from human brain tissue as previously described (DeSilva et al., 2007). Tissue was homogenized in a frosted glass homogenizer in 1% SDS containing a protease inhibitor cocktail with EDTA (Roche, Jena, Germany). After homogenization, samples were dispersed in an ultrasonic bath for approximately 15 minutes until the solution was clear. Protein concentration was measured using the Lowry assay with bovine serum albumin as the standard. Samples (40 µg/lane) were run on an 8–18% polyacrylamide gel and electroblotted onto a poly-vinylidene fluoride (PVDF) membrane (PerkinElmer, Wellesley, MA). PVDF membranes were incubated with anti-nGLT1 at 1 µg/ml overnight at 4°C in TBST buffer (50 mM Tris, 150 mM NaCl, 0.01% Triton, pH 7.4) containing 5% nonfat milk. Blots were then washed three times in TBST buffer followed by a 1-hour incubation with HRP-conjugated goat anti-rabbit IgG (Amersham Life Science, Fair Lawn, NJ). For protein detection, membranes were incubated in Western Lightning Chemiluminescence Reagent (PerkinElmer) and exposed on X-Omat Blue XB-1 film (Kodak). Densitometric analysis was performed on film using the MCID Elite version 7.0 software published by Imaging Research (St. Catharines, Ontario, Canada). A human adult standard lysate containing parietal white matter from three pooled cases (ages 55, 65, and 75 years) was run as a control on each blot. Density of the individual bands obtained from the human developmental series of cerebral white matter lysates was calculated and plotted as a percentage of the human adult standard, as performed in previous studies (DeSilva et al., 2007). A regression analysis of post-mortem interval (PMI) on EAAT2 expression in controls and in controls and PVL cases combined showed no statistically significant effect of PMI.

Immunocytochemistry

Standard immunocytochemistry methods were applied to deparaffinized tissue sections for the assessment of EAAT2 staining using the anti-nGLT1 antibody. After the tissue sections were deparaffinized, they were placed in 10 mM citrate buffer (pH 6.0) and boiled for 15 minutes to enhance antigenicity. Primary antibodies diluted in blocking buffer [phosphate-buffered saline (PBS) with 5% goat serum and 0.1% Triton-X] were placed on the tissue sections following a 1-hour incubation with blocking buffer at room temperature. Primary antibodies were incubated with the tissue sections overnight at 4°C. The secondary antibody (DakoCytomation, Carpinteria, CA) was then applied for 1 hour at room temperature, and the reaction was visualized with the chromagen 3,3'-diaminobenzidine (DAB). A light

hematoxylin counterstain was applied to each section. Negative control sections omitted the primary antibodies.

Double-labeling immunocytochemistry experiments were performed sequentially by staining the sections first with anti-nGLT1 antibody followed by the cell-specific markers (CD68, O4, and GFAP). EAAT2 was visualized with a secondary red fluorescent antibody (Alexa 594), whereas cell specific markers were visualized with a fluorescent green (Alexa 488) antibody. Specificity of secondary antibodies was confirmed by processing control sections without primary antibodies.

Quantitative analysis

Immunocytochemistry was performed on a subset of six PVL cases and six control cases to address the issue of the percentage of astrocytes (GFAP immunopositive) that express EAAT2, as determined by double labeling. After tissue sections were stained with the appropriate antibodies, photographs were taken of cerebral white matter in three different high-power fields (hpf); positively stained, double-labeled cells were counted by two observers, independently. The numbers of fluorescently labeled EAAT2-positive cells (Alexa 594, red), GFAP-positive cells (Alexa 488, green), and double-labeled EAAT2- and GFAP-positive cells (yellow) were counted. The number of EAAT2-immunopositive GFAP-labeled cells was divided by the number of GFAP-positive cells alone (Alexa 488 green) to yield the percentage of EAAT2-positive, GFAP-positive cells. The average percentage of the six PVL sections was compared with that from the six control sections using analysis of covariance (ANCOVA) models, adjusting for age. The number of GFAP (green)- and EAAT2 (red)-positive cells alone was also analyzed with ANCOVA models, adjusting for age.

Imaging

Digital imaging was performed on a Zeiss Axioscop equipped with a Spot advanced camera. Confocal imaging was performed on a Zeiss LSM 510 MetA microscope. Pictures were taken using Zeiss LSM software

Statistical analysis

An ANCOVA, adjusting for age, was performed to test for differences in EAAT2 protein expression between PVL and control cases. Standard error of the mean was used as a measure of variance between cases from the same age group. $P < 0.05$ was considered significant.

RESULTS

Clinicopathologic database

Tables 1 and 2 summarize the PVL ($n = 17$) and control ($n = 16$) cases used in the histopathologic and immunocytochemical analysis of this study. The mean PMI for PVL cases was 19 ± 5 hours and for control cases was 17 ± 3 hours (not significantly different).

Cellular pathology of PVL

The white matter pathology in the 16 PVL cases (Table 1A,B) was characterized by focal periventricular necrosis with different stages of organization and infiltration by macrophages (Fig. 1C), and diffuse reactive astrogliosis (Fig. 1A) and activated microglia (Fig. 1B) in the surrounding white matter, as demonstrated in a representative case.

Immunoblot analysis of EAAT2 in cerebral white matter tissue from PVL and control cases

The first goal of this study was to determine whether there is a quantitative difference in the expression of EAAT2 in PVL vs. control cases. The periventricular white matter in three PVL cases was assayed for EAAT2 protein expression using immunoblot analysis. These data were compared with data from 21 control cases analyzed for EAAT2 protein expression as previously described (De-Silva et al., 2007). A two-tailed *t*-test showed no significant difference in expression between PVL cases and control cases. Examination of the histograms of the three individual PVL cases plotted relative to their respective age-related controls, however, suggested a substantial increase in EAAT2 expression in the cerebral white matter in two PVL cases at 29 and 40 postconceptional weeks (Fig. 2). The PVL case at 29 weeks was 2.1 standard deviations above the mean of the controls in the 19–34-week range. The PVL case at 40 weeks was 3.4 standard deviations above the mean of the controls in the 38–41-week range. Because of the small sample size, we regard these data as anecdotal. Nevertheless, they suggest increased levels of EAAT2 in the cerebral white matter in PVL. Unfortunately, we were unable to obtain more frozen PVL tissue samples during the time frame of this study. However, we regarded these limited data as suggestive of increased expression of EAAT2 expression in PVL, warranting the cellular analysis performed below.

EAAT2 protein expression in reactive astrocytes

In the PVL cases ($n = 17$), histologic examination revealed diffuse reactive gliosis in the periventricular and deep white matter in the affected frontal, parietal, and occipital lobes peripheral to the periventricular foci of necrosis (see Fig. 1 for a representative case). In sections with single labeling for EAAT2, cells with the morphology of reactive astrocytes expressed EAAT2 throughout the diffuse white matter in the PVL cases (Fig. 3, arrows). The reactive astrocytes had enlarged nuclei and hypertrophied cytoplasm with prominent fibrillary processes. Scattered axons in the diffuse white matter were positive for EAAT2 expression in two of 17 PVL cases (Fig. 3, arrowheads) but not in any control cases. Using double-label immunocytochemistry, we determined that reactive astrocytes in PVL did indeed express EAAT2 (Fig. 4). From a subset of six PVL and six control cases, we also found that the percentage of GFAP-immunopositive astrocytes that express EAAT2 is significantly higher in the PVL cases compared with controls adjusted for age ($52\% \pm 5\%$ vs. $21\% \pm 5\%$; $P = 0.004$), suggesting that EAAT2 is up-regulated in the astrocyte population in PVL. Notably, there were very few GFAP-positive cells expressing EAAT2 in control tissue in the preterm cases, in direct contrast to the striking increase in GFAP-positive cells expressing EAAT2 in fetal PVL cases (Fig. 5). The density of reactive astrocytes in the PVL cases (46 ± 6 GFAP-immunopositive cells/hpf; $n = 6$) compared with controls (27 ± 6 immunopositive cells/hpf; $n = 6$) was marginally increased ($P = 0.07$) in this small subset, as was the number of EAAT2-immunoreactive cells in PVL (33 ± 4 /hpf; $n = 6$) compared with controls (19 ± 4 /hpf; $n = 6$; $P = 0.07$).

EAAT2 expression in macrophages/microglia in PVL

By using single-label immunocytochemistry, we observed, in the organizing necrotic foci of PVL, numerous cells with the morphology of macrophages that expressed EAAT2 (Fig. 6). Double-label immunocytochemistry with anti-nGLT1 (red) and CD68 (green) antibodies demonstrated that such macrophages did indeed express EAAT2 (Fig. 7). Cells with the morphology of resting or activated microglia (Fig. 1B) were not associated with EAAT2 expression in the PVL or control cases (data not shown).

EAAT2 expression in premyelinating oligodendrocytes

For PVL (n = 17) and control tissue (n = 16), we found cells staining for EAAT2 that had the morphology of developing OLs. The developing OLs were characterized morphologically in a representative 40-week PVL case (Fig. 8, arrow). Of note was that mature OLs (Fig. 8, arrowhead) did not stain for EAAT2, as reported for normative cases (DeSilva et al. 2007). With double-label immunocytochemistry, developing OLs expressed EAAT2 (green) and O4 (red) in the cerebral white matter of PVL and in control cases (Fig. 9). There was no obvious qualitative difference in EAAT2 expression in developing OLs in PVL and control cases.

DISCUSSION

The regulation of glutamate concentration in the extracellular space by glutamate transporters is essential for normal synaptic function (Danbolt, 2001) as well as neuronal survival by preventing excitotoxicity (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992; Rothstein and Tabakoff, 1994; Tanaka et al., 1997). Glutamate transporters are not ATP dependent; rather, their function is driven primarily by the transmembrane gradients of sodium and potassium (Danbolt, 2001). When there is a dissipation of electrochemical gradients across the plasma membrane as occurs during hypoxia-ischemia, glutamate transporters operate in reverse to release glutamate, thereby promoting excitotoxicity (Nicholls and Attwell, 1990; Pines and Kanner, 1990; Rossi et al., 2000; Szatkowski et al., 1990). The primary finding of our study was the marked expression of EAAT2 in reactive astrocytes in the diffusely gliotic white matter and in macrophages of the necrotic periventricular foci. Premyelinating OLs in PVL also expressed EAAT2, as previously demonstrated by us in the white matter from control human fetuses (DeSilva et al., 2007), but its expression was not different from that of controls. In two of three PVL cases in which frozen tissue was available to measure the overall EAAT2 level by immunoblotting, we found that the protein levels were at least two standard deviations above age-related controls. Thus, EAAT2 protein expression may be substantially increased in at least some cases of PVL, potentially at specific stages of the evolution of white matter damage. Our immunocytochemical data indicate that the overall increase in EAAT2 protein reflects a combination of factors, including an increase in the overall density and number of reactive astrocytes expressing EAAT2, an increase in expression of EAAT2 by individual astrocytes, and an increase in EAAT2 by macrophages that infiltrate the foci of periventricular necrosis. The up-regulation of EAAT2 by individual astrocytes is shown by our analysis demonstrating an increase in the percent of astrocytes expressing EAAT2 in PVL compared with controls by double-label immunocytochemistry. In the following discussion, we highlight the implications of our findings for understanding perinatal cerebral ischemia in humans.

The glutamate transporter EAAT2 and cerebral hypoxia-ischemia

EAAT2 plays an important role in hypoxia-ischemia in experimental models that is directly relevant for consideration of its role in PVL. GLT1 knockout mice are more vulnerable to neuronal loss in the hippocampus and attain higher extracellular glutamate levels in response to a 5-minute episode of ischemia than wild-type mice. After 20 minutes of ischemia, however, wild-type mice are more vulnerable to neuronal death and attain higher glutamate levels than knockout mice (Mitani and Tanaka, 2003). These data suggest that, with short episodes of ischemia, glutamate transport function is preserved, and glutamate transporters clear glutamate that has been released by other mechanisms; with prolonged ischemia, however, glutamate transporters appear to become a major source of abnormal extracellular concentrations of extracellular glutamate. In addition, animal studies have documented a loss or a redistribution of EAAT2 in neonates (Fukamachi et al., 2001; Pow et al., 2004) or

adults (Martin et al., 1997; Rao et al., 1998, 2001; Torp et al., 1995; Yi et al., 2004) within the first few days following hypoxia-ischemia, but the consequences of these changes in terms of cell injury are unknown. In the following sections, we consider the potential role of EAAT2 expression in pre-OLs, axons, reactive astrocytes, and macrophages in the pathogenesis of PVL.

Expression of EAAT2 in pre-OLs in PVL

Previously we showed that the glutamate transporter EAAT2 is up-regulated in human developing white matter in developing OLs (DeSilva et al., 2007) at a time when glutamate receptors are also present. This suggests that the developmental (normative) up-regulation of EAAT2 may contribute to the vulnerability of the immature white matter to excitotoxicity during the period of development when the fetus is at greatest risk for PVL. The potential role of excitotoxic damage to OLs in perinatal brain ischemia is well recognized (Volpe, 2001), emphasizing the need for carefully maintained glutamate homeostasis. This role appears to fall almost exclusively to developing OLs themselves in developing white matter because of the lack of EAAT2 expression in astrocytes (DeSilva et al., 2007). In culture, developing OLs [(O4⁺O1⁻MBP⁻) and (O4⁺O1⁺MBP⁻)] are sensitive to non-NMDA receptor-mediated excitotoxicity (Matute et al., 1997; McDonald et al., 1998; Rosenberg et al., 2003; Yoshioka et al., 1996). However, mature OLs (O4⁺O1⁺MBP⁺) are very resistant to excitotoxicity, because of down-regulation of glutamate receptors in this stage (Itoh et al., 2002; Rosenberg et al., 2003). The relevance of the in vitro studies is underscored by in vivo studies in which an AMPA receptor antagonist is protective against cerebral white matter damage in a perinatal rat model of selective white matter injury (Follett et al., 2000). NMDA receptors have recently been shown to be present on OL processes (Karadottir et al., 2005), in contrast to the localization of AMPA receptors, which are present on the OL soma. Overstimulation of AMPA receptors appears to lead to OL cell death, whereas overstimulation of NMDA receptors leads to loss of OL processes (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005).

Oligodendrocytes subjected to oxygen-glucose deprivation in vitro release glutamate by reversal of GLT1, insofar as dihydrokainate, a specific blocker of GLT1, is highly protective (Deng et al., 2003; Fern and Möller, 2000). We speculate, based on immunocytochemical analysis in the present study, that PVL does not affect the developmental expression of EAAT2 in OLs in human cerebral white matter. Because there is no obvious reduction in EAAT2, the mechanism to generate extracellular glutamate by reversed operation of transport on OLs remains intact. Notably, we observed axonal EAAT2 expression in a small sample of PVL cases (but not in controls) in the diffusely gliotic white matter. EAAT2 expression has been reported in corticofugal bundles in normative white matter in the developing human brain (Furuta et al., 2005). In animal models, EAAT2 is transiently expressed in axons at midgestation (Furuta et al., 1997; Northington et al., 1999; Yamada et al., 1998). EAAT2 expression in potentially damaged axons in human PVL, as suggested by this study, requires further investigation.

Expression of EAAT2 in resting and reactive astrocytes

Astrocytes in the mature brain play a critical role in regulating glutamate concentration at excitatory synapses via glutamate transporters that are heavily expressed at perisynaptic astrocytic processes (Lehre and Danbolt, 1998). Because of the expression of EAAT2, the predominant glutamate transporter, astrocytes are considered to be the most important cell type in maintaining glutamate homeostasis (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992). EAAT2 expression in excitatory presynaptic terminals has recently been demonstrated (Chen et al., 2004) and may also play an important role in glutamate clearance at synapses. Our previous data show that EAAT2 is not expressed in astrocytes in normative

cerebral white matter in humans until birth (DeSilva et al., 2007), consistent with previous studies in human brain tissue (Furuta et al., 2005) and cultured glial progenitors (Maragakis et al., 2004). Astrocytic expression of GLT1 also does not occur in rat white matter, mouse spinal cord, or sheep corpus callosum until after birth (Furuta et al., 1997; Northington et al., 1999; Yamada et al., 1998). In the present study, we confirmed our previous result showing that EAAT2 is not expressed in resting astrocytes in fetal cerebral white matter of control (normative) cases (DeSilva et al., 2007). Thus, its presence on reactive astrocytes in PVL in fetal cases of comparable postconceptional age (see below) is potentially an adaptive mechanism to assist in the maintenance of glutamate homeostasis in vulnerable white matter that has sustained injury.

Astrocytes become reactive in response to a host of insults to the central nervous system and can be potentially harmful based on their capacity to generate reactive nitrogen and oxygen species (Choi et al., 2004; Gibson et al., 2005; Halliwell and Gutteridge, 1998; Noh and Koh, 2000; Tolia et al., 1999a,b; Zaheer et al., 2004) or to form glial scars and thereby prevent axonal regeneration (Bush et al., 1999; Faulkner et al., 2004). Nevertheless, there is considerable evidence to support a protective role for reactive astrocytes (Faulkner et al., 2004). In an in vivo model of traumatic brain injury to the cerebral cortex, for example, transgenic mice in which reactive astrocytes expressing herpes simplex virus-thymidine kinase are ablated by treatment with gancyclovir demonstrate a 60% loss of cortical tissue compared with an 18% loss in control animals that do have reactive astrocytes (Myer et al., 2006). A similar in vivo study showed that reactive astrocytes protect tissue and preserve function in spinal cord injury (Faulkner et al., 2004). In contrast to the present study in PVL, astrocytes that are immunopositive for EAAT1 or EAAT2 decrease in number following traumatic brain injury in humans (van Landeghem et al., 2006). Alternately, an increase in EAAT1 in astrocytes has been reported in other human traumatic brain injury paradigms (Beschorner et al., 2007) and in prion disease (Chretien et al., 2004). If reactive astrocytes are stimulated to express EAAT2 after either hypoxia-ischemia or inflammation, perhaps the role of this up-regulation is to help in the removal of excess extracellular glutamate thought to be a key factor in the pathogenesis of PVL. The activation of EAAT2 in reactive astrocytes in PVL may be an adaptive mechanism designed to assist in the removal of excess glutamate that might occur following an initial insult. Injured white matter is likely to be at risk for subsequent episodes of hypoxia-ischemia. It is also possible that up-regulation of EAAT2 is due to secondary changes associated with gliosis rather than responsive to the primary insult itself.

Expression of EAAT2 in macrophages in PVL

EAAT2 and/or EAAT1 expression in macrophages has also been recognized in inflammation in several CNS diseases in which excitotoxicity has been implicated as a major mechanism of neuronal injury, including brain trauma (van Landeghem et al., 2006), HIV infection (Porcheray et al., 2006), and prion disease (Chretien et al., 2004). Relevant to CNS infection, lipopolysaccharide (LPS), the toxic component of the gram-negative bacterial wall, increases EAAT2 expression and function in macrophages/activated microglia and astrocytes in vitro (Nakajima et al., 2001; O'Shea et al., 2006; Persson et al., 2005; Rimaniol et al., 2000). We found in the present study that EAAT2 is expressed in macrophages in the periventricular necrotic foci in PVL tissue. These data suggest a role for macrophages, as well as reactive astrocytes, in assisting in the removal of excessive glutamate during the excitotoxic component of this disease.

CONCLUSIONS

In conclusion, excitotoxicity secondary to cerebral ischemia is considered a major potential mechanism of OL injury in PVL. In this study, we demonstrate activation of EAAT2

expression in astrocytes in PVL. This study highlights the need for further research into the role of glutamate transporters in the pathogenesis of perinatal white matter damage. The timing of the up-regulation of expression of EAAT2 in reactive astrocytes in response to the ischemic or inflammatory insult to white matter is not known. Animal models of PVL are needed to determine the role of EAAT2 in the pathogenesis of PVL. Of utmost importance is the possibility that glutamate transporters may be a therapeutic target at different stages in the clinical evolution of this disorder.

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Fig. 1. Neuropathological elements of PVL. Diffuse PVL in cerebral white matter from a 34-week-old case showing reactive astrocytosis (**A**), with DAB staining of anti-GFAP, and activated microglia (**B**), with DAB staining of CD68 counterstained with hemotoxylin. **C**: CD68 immunostaining counterstained with hemotoxylin in the same case showing macrophage infiltration in the focal necrosis of PVL. Scale bars = 50 μ m.

Fig. 2.

Densitometric analysis of EAAT2 protein expression in developing human cerebral white matter. Cerebral white matter lysates from cases of different ages were run on an SDS-PAGE (8–18%) with 40 μ g protein per lane and immunoblotted with anti-nGLT1 antibody. Densitometric values are plotted as percentage of adult human standard control.

Fig. 3. DAB staining of EAAT2 in PVL. Cerebral white matter from a 34-week PVL case showing EAAT2 staining in reactive astrocytes (arrows). Arrowheads point to laminar processes that appear morphologically axonal. Scale bar = 50 μ m.

Fig. 4.

Expression of EAAT2 in reactive astrocytes in cerebral white matter from PVL brains. Confocal fluorescence microscopy ($\times 60$) of reactive astrocytes in cerebral white matter from a 40-week-old infant diagnosed with PVL labeled with GFAP monoclonal antibodies (**A**) counterstained with anti-nGLT1 antibodies (**B**). Overlay of A and B shows EAAT2 labeling in reactive astrocytes (**C**); bisbenzamide for nuclear staining is blue. Scale bar = 50 μm .

Fig. 5.

Percentage of EAAT2-positive GFAP-expressing cells in PVL vs. control cases. The number of EAAT2-immunopositive GFAP-labeled cells was divided by the number of GFAP-positive cells alone to yield the percentage of EAAT2-positive, GFAP-positive cells. Scale bar = 50 μ m.

Fig. 6. Expression of EAAT2 in macrophages in cerebral white matter from PVL brains. DAB staining using anti-nGLT1 antibodies was performed on paraffin-embedded PVL tissue. Digital microscopy ($\times 40$) of cerebral white matter from a 40-week-old infant diagnosed with PVL labeled with anti-nGLT1 antibodies. Arrows show EAAT2-stained macrophages. Scale bar = 50 μm .

Fig. 7.

Expression of EAAT2 in macrophages in cerebral white matter from PVL brains.

Fluorescence microscopy ($\times 40$) of cerebral white matter from a 32-week-old preterm infant diagnosed with PVL labeled with anti-nGLT1 antibodies (**B**) counterstained with the CD68 monoclonal antibodies (**A**). Overlay of A and B shows EAAT2 labeling in macrophages (**C**). Scale bar = 50 μm .

Fig. 8.
DAB staining of EAAT2 in paraffin-embedded PVL human cerebral white matter. DAB staining of anti-nGLT1 in a 40-week PVL case. Arrows point to developing OLs staining with anti-nGLT1. Arrowheads indicate mature MBP OLs. Scale bar = 50 μ m.

Fig. 9.

Immunofluorescent double labeling of EAAT2⁺ and O4⁺ OLs in human developing cerebral white matter with PVL. Fluorescence microscopy ($\times 40$) of cerebral white matter in a 32-week-old preterm infant diagnosed with PVL labeled with the O4 monoclonal antibody (**A**) counterstained with anti-nGLT1 antibodies (**B**). Overlay of A and B shows EAAT2 expression in O4⁺ OLs (**C**). Scale bar = 50 μ m.

TABLE 1A

Summary of Cerebral White Matter Pathology in PVL Cases¹

Case	GA	PNA	PCA	Focal acute necrosis	Focal subacute necrosis	Focal scar w/mineral	Diffuse gliosis	↓ WM and/or ↑ vents
1	29	0	29	-	+	-	+	-
2	29	3	32	-	+	-	+	-
3	31	0	31	-	+	-	+	-
4	32	1	33	+	-	-	+	-
5	32	2	34	+	+	-	+	-
6	35	0	35	+	+	+	+	-
7	34	1	35	+	+	+	+	+
8	36	0	36	-	-	+	+	+
9	35	4	39	-	+	-	+	-
10	34	5	39	+	+	+	+	-
11	38	2	40	+	+	-	+	-
12	39	1	40	+	-	-	+	-
13	40	3	43	-	-	+	+	-
14	40	4	44	+	+	+	+	-
15	40	5	45	-	+	-	+	-
16	40	10	50	-	+	-	+	+
17	36	15	51	-	+	+	+	+

¹ +, present; -, absent; GA, gestational age in weeks; PNA, postnatal age in weeks; PCA, postconceptional age in weeks; mineral, mineralization; WM, white matter; Vents, ventricles. Prematurity is defined as a gestational age less than 37 weeks.

TABLE 1B

Continued Summary of the Neuropathology of PVL Cases¹

Case	BST	CB	BG	Thal	Hipp	CCH	WMH	SAH	GMH	Diagnosis
1	A	F	A	-	-	-	-	-	+	Skeletal dysplasia; uncertain type
2	C, G	A, B	A, B, G	A, B, G	-	-	-	+	+	Maternal cardiac arrest; emergent C-section
3	A	-	-	-	-	-	-	-	-	Prematurity; duodenal atresia
4	C	C	-	C	C	-	-	-	+	Prematurity
5	-	-	C	-	C	+	-	+	-	Prematurity
6	-	-	A, B	-	A	-	-	-	-	Prematurity; Potter's syndrome; abnormal temporal lobe gyration; malrotation of hippocampi; duplication of dentate gyrus
7	-	-	G	G	B	-	-	-	+	Prematurity
8	A	I	-	-	-	-	-	-	+	Prematurity; CMV infection in utero; ABO incompatibility; dwarfism/skeletal dysplasia; fetal heart failure
9	NA	J	A	A	A	K	+	-	-	Prematurity; twin; renal glomerular sclerosis with renal failure
10	A	-	-	-	-	-	-	+	+	Prematurity
11	A	F, K	A	A	A, B	D	-	+	-	Idiopathic primary pulmonary hypertension; ECMO treatment
12	-	A, C	A	A	-	B, C	-	-	-	Ornithine transcarbamylase deficiency
13	NA	NA	NA	NA	NA	NA	NA	NA	NA	Respiratory insufficiency; apnea; Hirschsprung's disease (Haddad syndrome)
14	A	A	-	A, B, M	A, B	-	-	-	-	Congenital heart disease; ECMO treatment
15	A	-	-	-	-	-	-	-	+	Marshall-Smith syndrome; respiratory stress
16	A, B	M	-	-	-	-	-	-	-	Hypoplasia of caudal medulla
17	-	-	-	N	-	-	-	-	-	Breech birth; oligohydramnios; pneumothorax; respiratory failure

¹ +, pathology present; -, pathology absent; A, reactive gliosis; B, neuronal loss; C, acute neuronal ischemia; D, hemorrhagic infarct; E, apoptosis; F, focal acute hemorrhage; G, mineralization; H, perivascular hemorrhage; I, microglial nodules; J, vermiform infarct; K, organizing infarct; L, Purkinje cell loss; M, microinfarct; N, necrotic focus and/or mineralization in hypothalamus; ECMO, extracorporeal membrane oxygenation; NA, not available; Bst, brainstem; CB, cerebellum; BG, basal ganglia; Thal, thalamus; Hipp, hippocampus; CCH, cerebral cortex hemorrhage; WMH, white matter hemorrhage; GMH, germinal matrix hemorrhage; PDA, patent ductus arteriosus.

TABLE 2A

Summary of Cerebral White Matter Pathology in Control Cases¹

Case	GA	PNA	PCA	Diffuse gliosis	↓ WM and/or ↑ vent
1	23	0	23	-	-
2	24	3	27	-	-
3	27	0	27	-	-
4	27	3	30	-	+
5	27	3	30	-	-
6	32	1	33	-	-
7	34	0	34	-	-
8	34	0	34	-	-
9	35	0	35	-	-
10	37	0	37	+	+
11	38	0	38	-	-
12	39	1	40	-	-
13	41	0	41	-	-
14	43	0	43	+	-
15	40	12	52	-	-
16	40	104	144	-	-

¹ +, present; -, absent; GA, gestational age in weeks; PNA, postnatal age in weeks; PCA, postconceptional age in weeks; mineral, mineralization; WM, white matter; Vents, ventricles. Prematurity is defined as a gestational age less than 37 weeks.

TABLE 2B

Continued Summary of the Neuropathology of Control Cases¹

Case	BST	CB	BG	Thal	Hipp	CCH	WMH	SAH	GMH	Diagnosis
1	-	-	-	NA	NA	-	-	-	-	Twin; extreme prematurity
2	-	-	-	-	-	-	-	+	+	Extreme prematurity
3	-	-	-	-	-	-	-	+	+	Extreme prematurity; pneumothorax
4	A	-	A	-	-	-	-	-	-	VACTERL association
5	A	-	-	-	-	-	-	+	-	Prematurity; NEC; sepsis
6	-	-	-	-	-	-	-	+	-	Extreme prematurity; tracheoesophageal fistula; respiratory distress
7	C	NA	C	NA	NA	C	-	-	-	Stillborn
8	A	-	-	-	-	-	F	-	-	Parvovirus B19; hydrops
9	-	O	-	-	NA	F	-	+	+	Alloimmune thrombocytopenia
10	C	C	C	-	-	C	-	-	-	Renal dysgenesis; Potter's sequence
11	A,B,C	C	NA	C	NA	C	-	-	-	Trisomy 21; no brain malformations
12	C	C	-	-	C	-	-	-	-	12:14 translocation; cardiac failure; coagulopathy; focal cortical dysplasia
13	-	-	-	-	-	-	-	-	-	Congenital heart disease; adrenal neuroblastoma
14	-	-	-	-	-	-	-	-	-	Pulmonary hypertension; pneumothorax
15	-	-	-	-	-	-	-	-	-	Hypoplastic left heart syndrome
16	-	-	-	NA	-	-	-	-	-	Acute ventricular fibrillation

¹ +, pathology present; -, pathology absent; A, reactive gliosis; B, neuronal loss; C, ischemic necrosis; F, focal microhemorrhage; NA, not available; Bst, brainstem; CB, cerebellum; BG, basal ganglia; Thal, thalamus; Hipp, hippocampus.

TABLE 3

Antibodies Used for Single- and Double-Label Immunocytochemistry and Western Blotting¹

Antibody	Source	Host and type	Antigen	Specificity	Dilution
GFAP (SMI 22)	Stemberger Monoclonals Lutherville, MD	Mouse mAb IgG1 (mixture)	Purified bovine GFAP	All components (clones 1B4, 2E1, 4A11) are specific for GFAP by radioimmunoassay, Western blot, and immunocytochemistry ^[1]	1:500
O4	Gift from Dr. S. Pfeiffer	Mouse mAb IgM	Bovine corpus callosum	Reacts selectively with OL surface antigens sulfatide and seminolipid on lipid immunodot blots ^[2,3]	1:500
CD68	Cell Marque	Mouse mAb IgG	Subcellular fraction of human alveolar macrophages	A highly glycosylated transmembrane protein expressed on endosomes and cell surface of activated microglia and macrophages ^[4] 1:50	
nGLT1	Rosenberg Lab	Rabbit pAb	Rat peptide containing aal-15 (M-A-S-T-E-G-A-N-N-M-P-K-Q-V-E)	Recognizes band on immunoblot of rat brain (67 kDa). Does not stain GLT1 knockout mouse using immunocytochemistry and immunoblotting ^[5,6] . Recognizes band on immunoblot of human brain (67 kDa). Preabsorption with the peptide used to generate the antibody showed no immunoreactivity, and no immunoreactivity was observed with the preimmune serum ^[7]	1:500

¹ aa, Amino acids; GFAP, glial fibrillary acidic protein; O4, OL progenitor surface markers; nGLT1, rat glutamate transporter homologue/EAAT2 in human. References:

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⁴ Billiards SS, et al. 2006. Development of microglia in the cerebral white matter of the human fetus and infant. *J Comp Neurol* 497:199-208.

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