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The Glutamate Transporter EAAT2 Is Transiently Expressed in Developing Human Cerebral White Matter

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Abstract

The major brain abnormality underlying cerebral palsy in premature infants is periventricular leukomalacia (PVL), a lesion of the immature cerebral white matter. Oligodendrocyte precursors (pre-OLs; $O4^{+}O1^{-}$) predominate in human cerebral white matter during the peak time frame for PVL (24–32 gestational weeks) and are vulnerable to excitotoxicity. We hypothesize that PVL reflects, in part, excitotoxicity to pre-OLs resulting from cerebral ischemia/reperfusion. Reversal of glutamate transport in the setting of energy failure is a major source of pathologic accumulation of extracellular glutamate. Here, we identify and localize the glutamate transporters in human cerebral white matter during the age range of PVL. In situ hybridization was performed with digoxigenin-labeled probes directed against the full-length coding regions of EAAT1, EAAT2, and EAAT3. EAAT2 mRNA was abundant in human fetal white matter during the period of peak incidence of PVL and virtually disappeared by 2 postnatal months. Its developmental profile differed significantly from that of both EAAT1 and EAAT3 mRNA. Immunoblotting demonstrated that EAAT2 protein was highly expressed in early development relative to adult values. Double-label immunocytochemistry detected EAAT2 in OLs but not astrocytes or axons in the human fetal white matter. We conclude that transient expression of EAAT2 occurs during the window of peak vulnerability for PVL, suggesting that this developmentally up-regulated transporter may be a major source of extracellular glutamate in ischemic injury to the cerebral white matter of the preterm infant. J. Comp. Neurol. 501:879–890, 2007.

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Keywords

cerebral palsy; excitoxicity; oligodendrocyte; hypoxic-ischemic injury; myelination; periventricular leukomalacia

Approximately 10–15% of babies born with birth weights less than 1,500 g develop cerebral palsy (Volpe, 2003). The major brain abnormality underlying cerebral palsy is periventricular leukomalacia (PVL), a disorder of the immature cerebral white matter hypothesized to result, at least in part, from cerebral ischemia/reperfusion. It is characterized by injury to premyelinating oligodendrocytes (pre-OLs) that appears to result in delayed or hypomyelination. The major risk period for PVL is at 24–32 gestational weeks, when pre-OLs predominate in human white matter (Back et al., 2001). Animal and cell culture data implicate excitotoxicity in injury to premyelinating OLs and thus have obvious relevance to understanding the cellular and molecular mechanisms underlying PVL (Deng et al., 2003; Follett et al., 2000; Itoh et al., 2002; Rosenberg et al., 2003).

Although excitotoxicity appears to be an important pathway of cell death in hypoxic-ischemic injury to white matter in the perinatal period, the source of the excess glutamate remains in question. During energy failure, glutamate transporters, which are physiologically responsible for clearing extracellular glutamate, operate in reverse and thereby release glutamate into the surrounding tissue (Deng et al., 2003; Fern and Möller, 2000; Roettger and Lipton, 1996; Rossi et al., 2000; Seki et al., 1999). 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 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). In adult mammalian brains, GLAST is expressed mainly in astrocytes and EAAC1 in neurons. The GLT1 transporter, previously thought to be expressed exclusively in astrocytes, is also expressed in neurons in at least two variant forms, GLT1a and GLT1b (Chen et al., 2002, 2004; Schmitt et al., 2002). In experimental animals, GLT1a protein is transiently expressed in growing axons in rat white matter, mouse spinal cord, and sheep corpus callosum, before the establishment of expression in astrocytes postnatally (Furuta et al., 1997; Northington et al., 1999; Yamada et al., 1998). Although studies of human adult brains have shown GLT1a expression in astrocytes and most recently in OLs (Bar-Peled et al., 1997; Milton et al., 1997; Pitt et al., 2000), there is no information about glutamate transporter expression in developing human cerebral white matter.

We hypothesized, in light of the developmental expression of GLT1 in central white matter axons in animals, that the human homologue EAAT2 is similarly elevated in early life, particularly during the peak period of vulnerability to PVL, and declines to adult levels thereafter. A transient expression of GLT could account, at least in part, for the vulnerability of immature cerebral white matter to ischemic injury. To test this hypothesis, we characterized glutamate transporter expression in the developing human cerebral white matter of autopsy cases without significant white matter pathology by using in situ hybridization, quantitative immunoblot analysis, and immunocytochemistry.

MATERIALS AND METHODS

Clinical database

Tissue was accrued from the cerebra of human fetuses and infants from a standardized level, i.e., parietooccipital lobe at the level of the occipital horn of the lateral ventricle, and from the body of the corpus callosum (level of posterior frontal white matter). Tissue samples were obtained through the Departments of Pathology, Brigham and Women's Hospital and Children's Hospital (Boston, MA), with parental and institutional approval. Parietooccipital white matter was also obtained from adult patients without neurological disease (21–75 years), as an index of maturation for comparison. The age of the infant cases is expressed in postconceptional (gestational plus postnatal) weeks (Table 1). Only samples with a post-mortem interval of less than 24 hours was used.

At autopsy, the parietooccipital poles were removed from cases and snap frozen immediately in blocks at –70°C for in situ hybridization (19 cases) and immunoblot assay (21 cases), or were immersed in freshly prepared 4% paraformaldehyde (Sigma, St. Louis, MO) in phosphate-buffered saline for immunocytochemistry (seven cases). Adjacent blocks were paraffin embedded for histopathologic assessment. Standard neuropathologic evaluations of cerebral cortex, white matter, diencephalon, hippocampus, brainstem, and cerebellum were performed in each case.

In situ hybridization

Frozen brain tissue was sectioned at 15 mm on a Leitz cryostat and mounted on glass slides. Plasmids (gifts of Dr. Susan Amara) containing the cDNAs of EAAT1, EAAT2, and EAAT3 (GenBank accession Nos. NM_004172, NM_004171, and NM_004170, respectively) were used to amplify by PCR the sense and antisense full-length coding regions of each transporter with primers that contained RNA polymerase initiation sites. Analyses of EAAT1 and EAAT3 mRNA were performed to determine the differential patterns of expression of the other transporters relative to EAAT2, the focus of this study. The amplified cDNA was used for transcription to generate digoxigenin (DIG)-labeled cRNA probes for each transporter. After transcription, all probes were alkali hydrolyzed to an average length of 500 base pairs. Nonisotopic in situ hybridization was performed according to a previously described method (Berger et al., 2005; Berger and Hediger, 1998; Chen et al., 2004), which is an improved method based on a previously published protocol (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, in situ hybridization probes were labeled with DIG. After hybridization, sections were incubated with a sheep anti-DIG-Fab antibody fragment conjugated to alkaline phosphatase and developed with 5-bromo-4 chloro-indoylphosphate and nitro blue tetrazolium (NBT/BCIP). As a negative control, adjacent sections were stained with a noncomplementary sense probe. A regression analysis of post-mortem interval (PMI) on EAAT1, EAAT2, and EAAT3 expression in control cases showed no statistically significant effect of PMI.

Cell counting from tissue sections labeled by using in situ hybridization

Counting of transporter-positive cells/high-power field (hpf) in single-labeled tissue sections was performed. Positive cells per hpf at "400 magnification (0.173 mm^2) were counted in

an area representative of all fields. The number of positive cells containing mRNA per hpf (cell/hpf) are reported in Figure 3 after being subjected to the post hoc correction factor for cell size used by Abercrombie (1946) and Guillery (2002). The thickness of all sections was 15 μm. The diameter of 20 cells from two different human cases for each probe was measured with a Nikon E800 digital microscope with Spot camera software. The average cell diameter in tissue stained for each probe was 11.7 ± 0.65 μm (EAAT1), 11.6 ± 0.87 μm (EAAT2), and 11.3 ± 1.0 μm (EAAT3), giving a correction factor of 56–57% for all probes. Two observers scored each case together, without knowledge of case diagnosis or age.

Northern blot analysis of RNA probes on fetal brain lysates

The specificity of the in situ probes was tested by Northern blot analysis based on a method described previously (Sambrook and Russell, 2001). Briefly, fetal human forebrain total RNA (Stratagene, La Jolla, CA) at 10 μg/lane was separated by electrophoresis on a 0.9% agarose-formaldehyde gel, electroblotted onto a charged cellulose membrane, and hybridized to ^{32}P-labeled RNA probes generated against the full-length coding regions of EAAT1, EAAT2, and EAAT3. ^{32}P-UTP-labeled probes were generated with a Maxiscript kit from Ambion (Austin, TX). The sizes of the glutamate transporter transcripts were determined by using a standard curve prepared from the 18S and 28S ribosomal RNA bands and RNA molecular weight markers (Invitrogen, Carlsbad, CA).

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) and has been previously characterized (Chen et al., 2002, 2004). This antibody was generated based on the published sequences for rat GLT1a and GLT1b (aa 1–15; GenBank accession No. AF451299), which are identical to each other and to the human sequence, and thus was used to detect EAAT2a and EAAT2b in human tissue in this study. To determine the specific cell types in which glutamate transporters are located, the following antibodies were used for double-labeling immunocytochemistry: anti-GFAP for astrocytes (Sternberger Monoclonals, Lutherville, MD), antivimentin (Chemicon, Temecula, CA) for radial glia, A2B5 monoclonal antibody for glial progenitors [grown from cells provided by the American Type Culture Collection (ATCC)], O4 monoclonal antibody for precursor OLs, O1 monoclonal antibody for immature OLs, antimyelin basic protein (MBP) for mature OLs (Sternberger Monoclonals), antiNeuN (Chemicon) for neuronal cell bodies, SMI-312 (Sternberger Monoclonals), and GAP-43 for axons. Hybridoma cells producing O4 and O1 monoclonal antibodies were generously provided by Dr. Stephen Pfeiffer. A summary of antibodies used in this study is included in Table 2.

Immunoblot analysis of human fetal brain lysates

Lysates were made from human brain tissue. Briefly, tissue was homogenized in a frosted glass homogenizer in 1% sodium dodecyl sulfate (SDS) containing a protease inhibitor cocktail with EDTA (Roche). After homogenization, samples were dispersed in an ultrasonic bath for approximately 15 minutes until the solution was clear. Protein concentration was measured using the DC Protein Assay (Bio-Rad, Hercules, CA). Samples (40 μg/lane) were run on an 8–18% polyacrylamide gel and electroblotted onto a polyvinylidene fluoride

(PVDF) membrane (Perkin Elmer, 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 horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Amersham Life Science, Piscataway, NJ). For protein detection, membranes were incubated in Western Lightning Chemiluminescence Reagent (Perkin Elmer) 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 (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. A regression analysis of PMI on EAAT2 expression in controls and in controls and PVL cases combined showed no statistically significant effect of PMI.

Immunocytochemistry

Immunocytochemical studies were performed on 4% paraformaldehyde-fixed tissue that was cryoprotected with 30% sucrose. Tissue was sectioned on a freezing vibratome and prepared as 50-μm floating sections. Briefly, sections were washed in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 2 mM potassium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) with 0.1% Triton X-100 (except when using O4 and O1 monoclonal antibodies against surface markers that do not require permeabilization) before blocking with 5% goat serum. Sections were incubated with primary antibodies overnight, followed by washing in PBS. Primary antibody labeling of tissue sections was detected with immunofluorescent secondary antibodies: Alexa 594 (red) and Oregon 488 (green; Molecular Probes, Eugene, OR). Sections were mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL) with added bisbenzamide to identify cell nuclei. Slides were observed with a Nikon E800 microscope, and photographs were taken with a Spot digital camera.

Statistical analysis

To assess the relationship between the mRNA and protein expression of each transporter and age, linear and quadratic regression analyses were performed. To determine whether levels differed before and after term birth (40 weeks), Wilcoxon rank sum tests were utilized. To test the EAAT2 protein expression (as percentage of adult standard) against the adult standard, representing 100% expression, a one-sample *t*-test was used. The measure of dispersion used throughout Results is the standard error of the mean.

RESULTS

Clinicopathological data set

Table 1 shows the cases used for the ensuing studies: 19 cases for in situ hybridization, 21 for immunoblot analysis, and seven for immunocytochemical analysis. They had died of a variety of disorders, but standard neuropathologic and histopathogic examination in each case did not reveal diagnostic cerebral white matter damage or other CNS abnormalities.

Specificity of glutamate transporter mRNA probes for in situ hybridization

The specificity of glutamate transporter mRNA probes used for in situ hybridization was tested by Northern blot analysis. The probes were transcribed from the same full-length coding regions of the transporters used for in situ and were labeled with ^{32}P. Each mRNA probe detected a distinct mRNA transcript for its respective glutamate transporter subtype in human fetal brain total RNA. The EAAT2 probe hybridized to a 11.7-kb band, the EAAT1 probe hybridized to a 4.0-kb band, and the EAAT3 probe hybridized to a 3.7-kb band (Fig. 1). Similar results were obtained with cDNA probes used to detect glutamate transporters in total brain RNA prepared from human cases ranging in age from 16 to 75 years (Nakayama et al., 1996).

In situ hybridization of EAAT1, EAAT2, and EAAT3 mRNA in developing human cerebral white matter

Analysis of mRNA expression was performed via in situ hybridization in 19 baseline cases, without neurological disease or significant brain histopathology, with probes against the full-length coding region of EAAT1, EAAT2, and EAAT3. Tissue was available from seven fetuses, seven infants, one child, and four adults (for comparison), although tissue was not available from all cases for each probe (Table 1). The sense cRNA probes were used as control probes for all three transporters, and did not yield any staining. The developmental profiles for the different transporters varied from one another substantially (Figs. 2, 3). EAAT2 mRNA expression in the parietooccipital white matter was low at midgestation; increased to its highest level of expression at 25 and 32 gestational weeks, i.e., the peak age window of PVL (Fig. 2C); and declined after 50 postconceptional weeks, i.e., 1.5 postnatal months to reach low adult levels between 55 and 74 postconceptional weeks (Figs. 2D, 3B). EAAT1 expression increased across the last half of gestation between 25 and 32 postconceptional weeks (Fig. 2A), the earliest time points examined, until approximately 50 postconceptional weeks, i.e., 1.5 postnatal months, and remained level at high adult values (Figs. 2B, 3A). EAAT3 mRNA expression was low at midgestation (Fig. 2E) and increased linearly between 34 and 40 postconceptional weeks to an intermediate level as seen in adults compared with EAAT2 and EAAT3 (Figs. 2F, 3C). We also considered the glutamate transporter mRNA levels relative to term birth (40 gestational weeks), because the peak age of vulnerability of PVL is in the mid- to late fetal period of pregnancy (Fig. 4). The differences before and after term birth were significant for all three transporters, giving the following P values determined from Wilcoxon tests: EAAT1 (0.003), EAAT2 (0.014), and EAAT3 (0.003). EAAT1 and EAAT3 mRNA levels were higher after than before birth; in contrast, EAAT2 mRNA levels were higher before than after birth (Fig. 4).

Evaluation of EAAT2 protein expression in the human fetus and infant

Because the in situ results (Figs. 2, 3) demonstrated that EAAT2 is developmentally up-regulated during the peak of vulnerability for premature infants to PVL, we analyzed the expression of EAAT2 at the protein level by using an antibody raised against the N terminus of GLT1/EAAT2, known to be shared by the two known variant forms of GLT1/EAAT2. To test the specificity of the antinGLT1 antibody in human brain tissue, immunoblot analysis was performed on lysates prepared from normal human motor cortex

(Fig. 5). A dense band appeared at 67 kDa (Fig. 5, lane 2). A light band was also observed above the 120-kDa marker (Fig. 5, lane 2), probably representing dimers of the transporter. These data are consistent with results obtained with this antibody in rodent brain lysates (Chen et al., 2002, 2004). To demonstrate the specificity of the immune response to the antigenic N-terminus GLT1 peptide, the immunoreactivity of purified anti-nGLT1 antibody was compared with that of the preimmune serum (Fig. 5, lane 1). In addition, the ability of the peptide against which the antibody was generated to block immunoreactivity was tested (Fig. 5, lane 3). Preimmune serum was not immunoreactive (Fig. 5, lane 1). Antigenic peptide nearly completely blocked the immunoreactivity of the anti-nGLT1 antibody against human brain lysate (Fig. 5, lane 3). Thus, the anti-nGLT1 antibody specifically detected protein containing the epitope against which it was generated in human lysates. Previous studies have shown that the immunreactivity produced by this antibody is not present in brain lysates or in tissue sections from GLT1 knockout mice (Chen et al., 2004).

To assess whether the transient expression of EAAT2 mRNA occurs at the protein level, an immunoblot using anti-nGLT1 antibody in parietooccipital cerebral white matter lysates was performed (Fig. 6). Lane 1 (Fig. 6) contains adult human standard cerebral white matter lysate (see Materials and Methods). Lane 2 (Fig. 6) contains white matter from a 30-week-old preterm infant. Comparison of lanes 1 and 2 (Fig. 6) shows a substantially increased expression of EAAT2 protein at 30 weeks compared with the adult. Lane 3 (Fig. 6) contains cerebral gray matter from the same 30-week-old preterm infant and shows that at this age the expression of EAAT2 in the cerebral white matter is comparable to the expression in the cerebral gray matter. Another example of cerebral white matter from a 30-week-old preterm infant is shown in lane 4 (Fig. 6), demonstrating similar expression to the case shown in lane 2 (Fig. 6).

To quantitate EAAT2 protein expression across human development, using densitometric analysis, lysates taken from parietooccipital white matter at different postconceptional ages were immunoblotted with anti-nGLT1 antibody. Data are expressed as percentage of adult human standard, run on each blot. White matter samples were obtained from 20 fetuses and infants and from one adult, aged 21 years (Table 1). Comparison of protein expression of EAAT2 at different ages is shown in Figure 7 and grouped according to biologically relevant epoch; 19–34 weeks, preterm; 38–41 weeks, term; 1–6 months, infant; 1–3 year, child; 21–75 years, adult. A linear regression showed no statistically significant change of EAAT2 protein expression across age. For the group of cases at 19–41 weeks, however, the mean densitometric value was 614% ± 37% of the 100% adult (standard) level. This value was significantly higher than the adult standard value (onesample *t*-test; P% 0.001).

Immunocytochemistry of EAAT2 in fetal human white matter

To determine the cellular localization of EAAT2 expression in the developing human white matter, we performed single- and double-label immunocytochemistry on seven human cases (Table 1) Relatively little EAAT2 staining was present in the cerebral white matter at 3 years of age compared with a 31-week-old case (data not shown). Cerebral cortex (CTX) staining was present in both cases and served as a positive internal control. To identify cellular elements expressing EAAT2, double-labeling experiments with anti-nGLT1 antibody and

cell-specific markers were performed. Fluorescence microscopy of the parietooccipital white matter (Fig. 8) from a representative case at 31 gestational weeks demonstrated anti-GFAP labeling of astrocytes (Fig. 8A), vimentin labeling of radial glia (Fig. 8C), and neurofilament labeling of axons (Fig. 8E). Double labeling showed no colocalization of EAAT2 with GFAP (Fig. 8B), vimentin (Fig. 8D), or neurofilament (Fig. 8F). In contrast, in pre-OLs in a representative 23-week case (midgestation) colocalization was seen with the antinGLT1 antibody (Fig. 9A) and the O4 monoclonal antibody (Fig. 9B). Coincidence of labeling with the anti-GLT1 and O4 antibodies is indicated by the appearance of yellow labeling of the overlying images (Fig. 9C).

DISCUSSION

In this study, we identify and localize glutamate transporters in the cerebral white matter in the human fetus and infant. Our main finding is that EAAT2 mRNA, as demonstrated by in situ hybridization, is abundant in human fetal white matter during the period of peak incidence of PVL and is substantially down-regulated at a time when myelination is initiated postnatally. The developmental profile of EAAT2 differed considerably from that of both EAAT1, which increased significantly during development and remained highly expressed through adulthood, and EAAT3, which was expressed at relatively low levels during pre- and postnatal development. Immunoblotting demonstrated that EAAT2 protein was highly expressed in early human development relative to adult values, and double-label immunocytochemistry detected EAAT2 in $O4^+$ OLs. The transient expression of EAAT2 coincides with the window of vulnerability for PVL, suggesting that reversal of this transporter might be a major source of glutamate in hypoxic-ischemic injury to the cerebral white matter of the preterm infant.

Pathophysiological implications of upregulation of EAAT2 in developing cerebral white matter

The role of glutamate excitotoxicity in hypoxic-ischemic injury to the neonate has been suggested clinically by the elevation of glutamate in the cerebrospinal fluid of term infants after perinatal hypoxia-ischemia (Hagberg, 1992) and experimentally by in vivo microdialysis in a perinatal rat model of ischemia (Benveniste et al., 1984; Silverstein et al., 1991) and, more recently, in a fetal sheep model (Loeliger et al., 2003). In culture, pre-OLs ($O4^+O1^-MBP^-$ and $O4^+O1^+MBP^-$) are sensitive to AMPA/kainate 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 resistant to excitotoxicity (Rosenberg et al., 2003) 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 suggested by in vivo studies in which an AMPA receptor antagonist was protective against cerebral white matter damage in a perinatal rat model of selective white matter injury (Follett et al., 2000, 2004), and calcium-permeable AMPA receptors were shown to be upregulated in cerebral white matter during the period of peak vulnerability to PVL (Follett et al., 2003, 2004; Talos et al., 2005a,b). Furthermore, recent data have shown that NMDA receptors are also expressed in developing oligodendrocyte processes and mediate injury (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). Although excitotoxicity may be a

fundamental mechanism in the pathogenesis of PVL, the source of the excess extracellular glutamate remains in question. The results presented herein suggest that part of the basis of the maturation-dependent vulnerability of white matter is due to the transient up-regulation of EAAT2.

Glutamate transporters provide the only known mechanism for maintaining extracellular glutamate homeostasis (Danbolt, 2001; Tanaka et al., 1997). Control of extracellular glutamate concentration is disrupted under conditions leading to excitotoxic injury (Lipton and Rosenberg, 1994; Meldrum and Garthwaite, 1990). When there is a dissipation of electrochemical gradients across the plasma membrane, such as occurs during hypoxiaischemia, glutamate transporters effectively act in reverse to release glutamate (Nicholls and Attwell, 1990; Szatkowski et al., 1990). In addition, OLs subjected to oxygen-glucose deprivation in vitro have been shown to 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). 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. However, after 20 minutes of ischemia, wild-type mice are more vulnerable to neuronal death and attain higher glutamate levels than knockout mice (Mitani and Tanaka, 2003). Thus, whether expression of EAAT2 in developing human white matter is helpful or harmful may depend on the duration of the ischemic episode. Presumably, in the Mitani and Tanaka experiments, prolonged ischemia compromised energy metabolism sufficiently to induce release of glutamate from cells through their glutamate transporters. Taken together, these data suggest that the upregulation of EAAT2 in developing OLs may be the source of excessive extracellular glutamate (by reverse transport) that overstimulates glutamate receptors, also present on OLs at this time, leading to excitotoxicity. Other possible sources of excessive extracellular glutamate accumulation, besides glutamate transporters, are synaptic release of glutamate from neurons; release through swellingactivated anion channels in astrocytes (Kimelberg et al., 1990; Takano et al., 2005) or hemichannels in activated microglia (Takeuchi et al., 2006); and exocytotic release from astrocytes mediated by chemokines (Bezzi et al., 2001), prostaglandins (Bezzi et al., 1998), and neuropeptides (Parpura et al., 1994). Although there are several possible sources of extracellular glutamate, glutamate transport is the only known clearance mechanism. Therefore, any disturbance in glutamate transport, such as down-regulation or blockage of uptake by cytokines (Fine et al., 1996; Hu et al., 2000; Liao and Chen, 2001; Ye and Sontheimer, 1996), reactive oxygen species (Volterra et al., 1994), or reactive nitrogen species (Trotti et al., 1996), will impair glutamate homeostasis.

Glutamate uptake in OLs may be critical to glutamate homeostasis in developing cerebral white matter

The present data show that astrocytes in human cerebral white matter during the peak age of vulnerability for PVL do not express EAAT2. Previously, no astrocytic expression of EAAT2 protein was found in the developing human CNS (Furuta et al., 2005) or in human cultured glial progenitors (Maragakis et al., 2004). Astrocytic expression of GLT1 was also not observed in rat white matter, mouse spinal cord, and sheep corpus callosum until postnatal development (Furuta et al., 1997; Northington et al., 1999; Yamada et al., 1998).

Our data show that EAAT2 is abundant in developing human white matter and is located on pre-OLs when the white matter is most vulnerable to PVL. It has been assumed that astrocytes have the major role in maintaining glutamate homeostasis (Bergles et al., 1999; Danbolt, 2001; Rosenberg and Aizenman, 1989; Tanaka et al., 1997). In fetal human white matter, oligodendroctyes appear to have an important role in glutamate homeostasis, in that pre-OLs and not astrocytes are the site of expression of EAAT2.

We did not observe the localization of GLT1/EAAT2 protein in growing axons that has been observed in studies of developing rodent and sheep brain. One explanation for this observation is that EAAT2 is transiently expressed in human axons during the fetal period at an earlier time (>23 weeks) than was included in our clinical database. This is suggested by one study that showed partial coexpression of EAAT2 and (-tubulin in the area of corticofugal bundles adjacent to radial fibers in the intermediate zone only at 20 GW in the human fetus (Furuta et al., 2005). The absence of EAAT2 from axons and astrocytes during the period of maximal vulnerability to PVL and the presence of EAAT2 in pre-OLs during this period suggest that pre-OLs may make a significant contribution to glutamate homeostasis during development.

Physiological implications of up-regulation of EAAT2 in the cerebral white matter of the human fetus and infant

A key physiological question concerns the significance of the developmental up-regulation of EAAT2 in the human white matter in early life. This up-regulation occurs at a time when pre-OLs express glutamate receptors, and the coincidence of these two phenomena suggests that glutamate receptor activation is important in the development and function of these cells. The expression of both glutamate receptors and transporters in pre-OLs also strongly suggests that there is a source for glutamate in developing white matter that causes activation of the OL glutamate receptors. Possible sources include astrocytes (Bezzi et al., 1998, 2001; Kimelberg et al., 1990; Parpura et al., 1994), microglia (Nakamura et al., 2003), and growing axons (Li and Stys, 2001; Lin et al., 2005). The functional consequences of glutamate receptor activation may be regulation of OL proliferation and differentiation, migration, and/or myelination (Gallo et al., 1996; Yuan et al., 1998). Glutamatergic synapses on oligodendroglial progenitors have been shown in the hippocampus (Bergles et al., 2000). It has become increasingly evident that developing oligodendrocytes are a target for glutamate signaling; therefore, further study is warranted focusing on the regulation of glutamate transporter expression and function in OLs.

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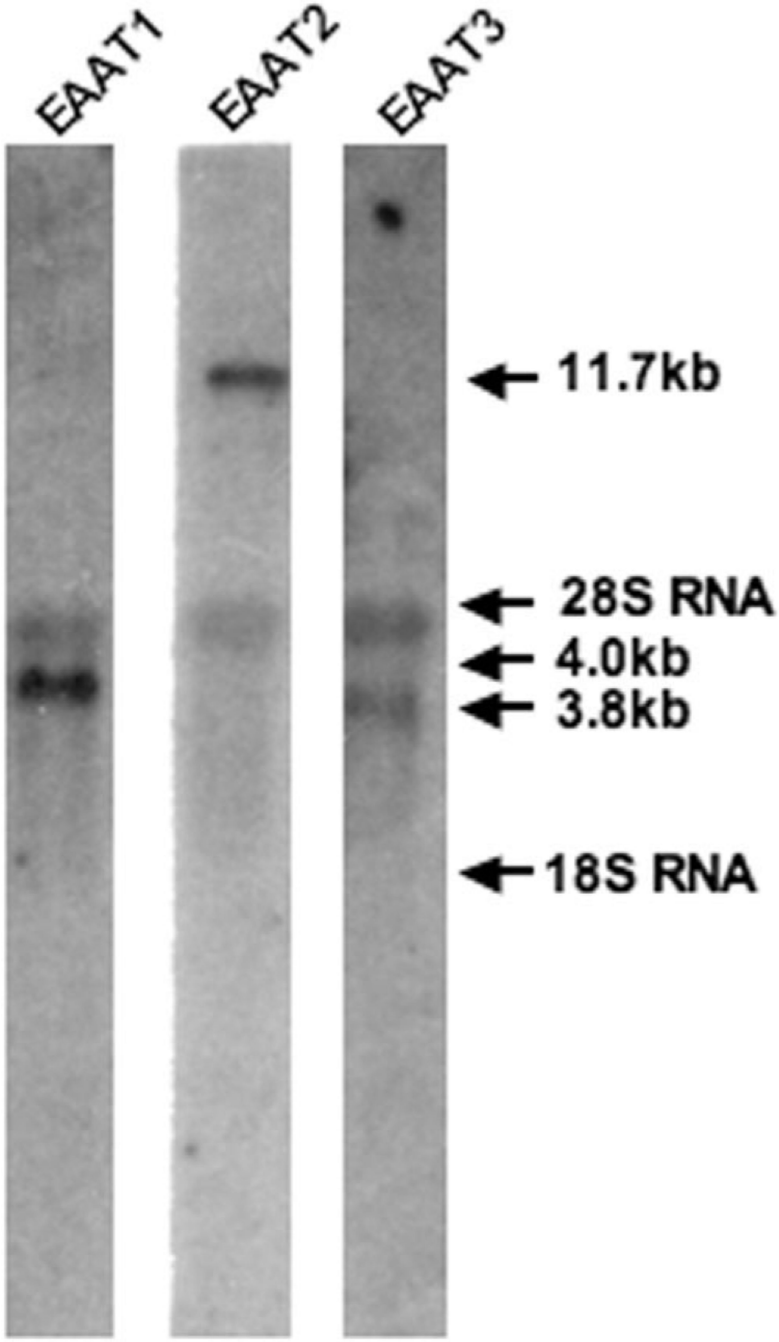

Fig. 1.

Northern blot analysis of glutamate transporter mRNA expression in total RNA extracted from human fetal brain tissue. Ten micrograms of fetal total RNA were loaded per lane. ^{32}P-labeled RNA probes were generated against the full-length coding regions of EAAT1, EAAT2, and EAAT3 and hybridized to RNA at 4.0, 11.7, and 3.7 kb, respectively. 28s RNA is also observed at 4.5 kDa.

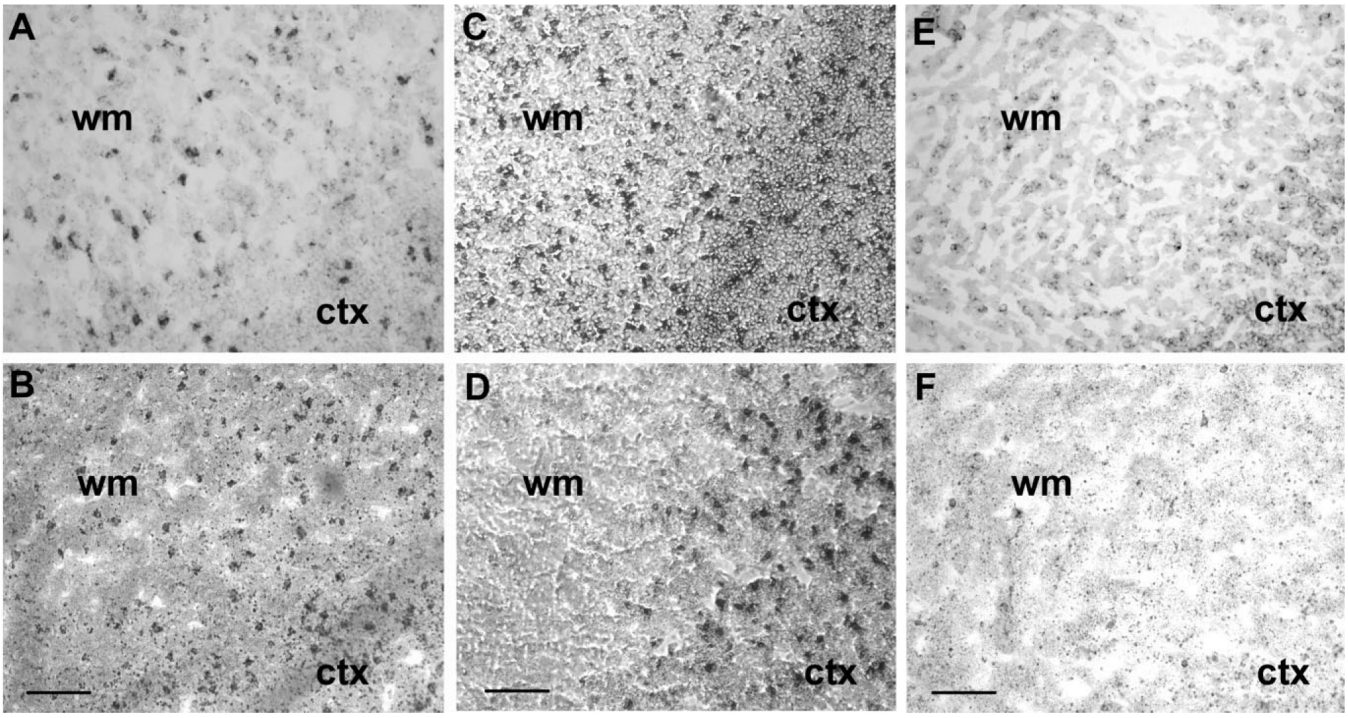

Fig. 2.

Nonisotopic in situ hybridization of EAAT2/GLT1, EAAT1/GLAST, and EAAT3/EAAC1 mRNA in human fetal cerebral white matter. **C** shows a section of white matter (WM) and cortex (CTX) of a 32-week-old subject, demonstrating a prominent amount of EAAT2 mRNA in the white matter. In contrast, a 7-month-old subject (**D**) expresses almost no EAAT2 mRNA in the white matter. Cortex serves as a positive control, showing EAAT2 labeling in both subjects. EAAT1 (**A,B**) and EAAT3 (**E,F**) expression was present and increased with development, in contrast to EAAT2. Scale bars &50 μm.

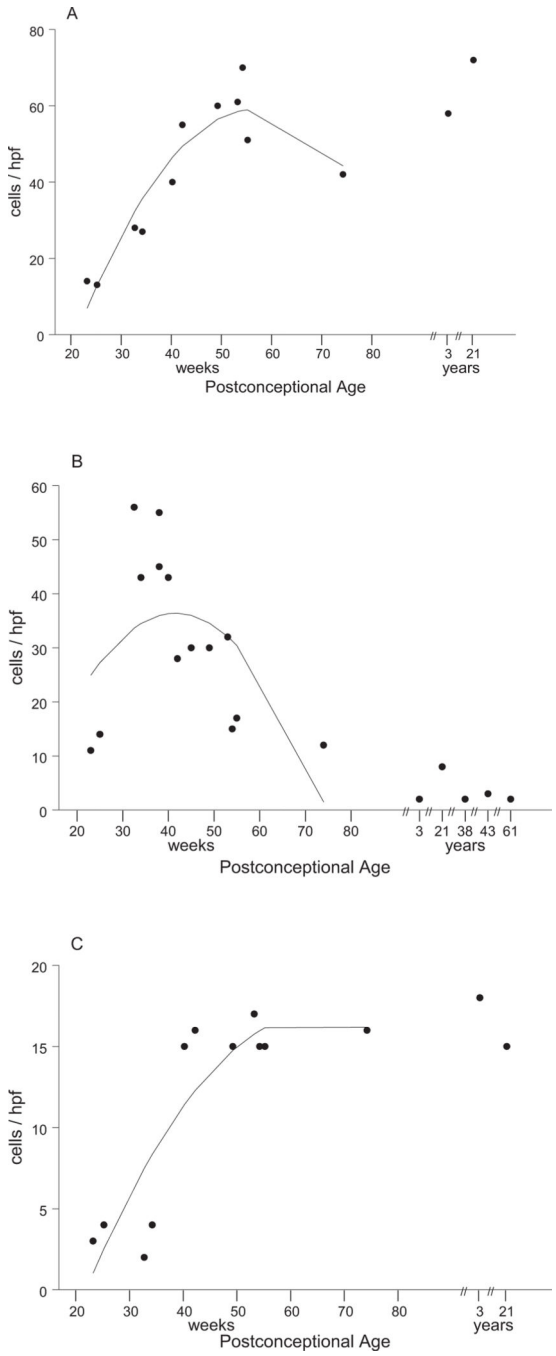

Fig. 3.

EAAT1, EAAT2, and EAAT3 expression as a function of age. A regression analysis of the effect of age on mRNA expression for each glutamate transporter is indicated by the curve drawn through each plot of individual data points. These data show that EAAT1 increases quadratically with age. EAAT2 changes quadratically with age, first increasing, then decreasing. EAAT3 increases with age. Regression analysis did not include ages more than 1 postnatal year, because the large variation in ages of older cases would influence the regression analysis.

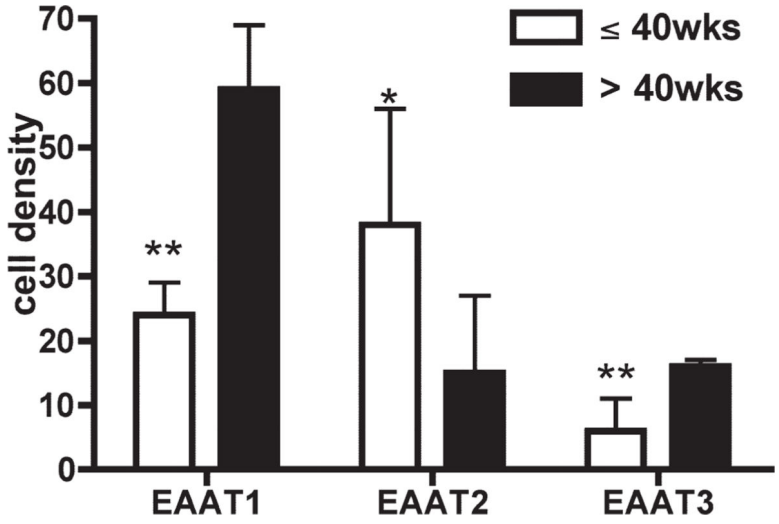

Fig. 4.

Comparison of preterm (≤40 weeks) and postterm (>40 weeks) expression of EAAT1, EAAT2, and EAAT3 mRNA. EAAT1 and EAAT3 levels were significantly higher after term, whereas EAAT2 levels were significantly lower after birth. Statistical analysis of preterm and postterm expression using the Wilcoxon test gives *P* values of 0.003 (EAAT1), 0.014 (EAAT2), and 0.003 (EAAT3). **P* % 0.05, ***P* % 0.01.

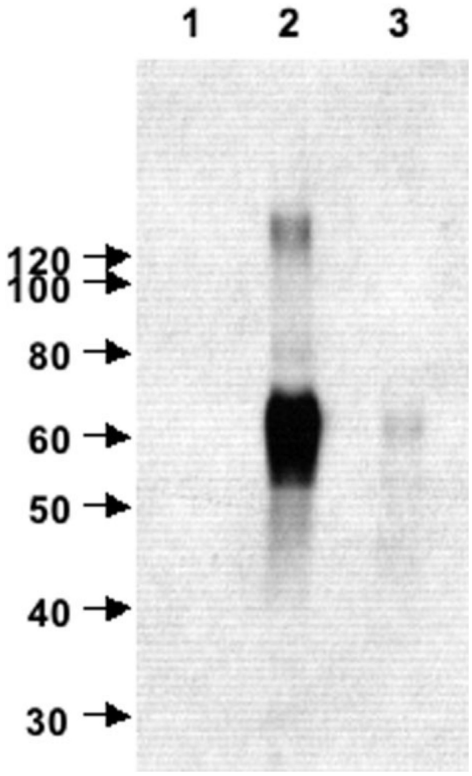

Fig. 5.

Western blot analysis of the specificity of the anti-nGLT1 antibody in human brain tissue. To characterize the anti-nGLT1 antibody, it was tested on lysates prepared from normal human motor cortex and appears as a band at 67 kDa (lane 2). A lighter band is also observed above the 120-kDa marker (lane 2). To demonstrate the specificity of the immune response to the nGLT1 peptide, the immunoreactivity of the anti-nGLT1 antibody was compared with that of the preimmune serum (lane 1). In addition, the ability of the peptide against which the antibody was generated to block immunoreactivity was tested (lane 3).

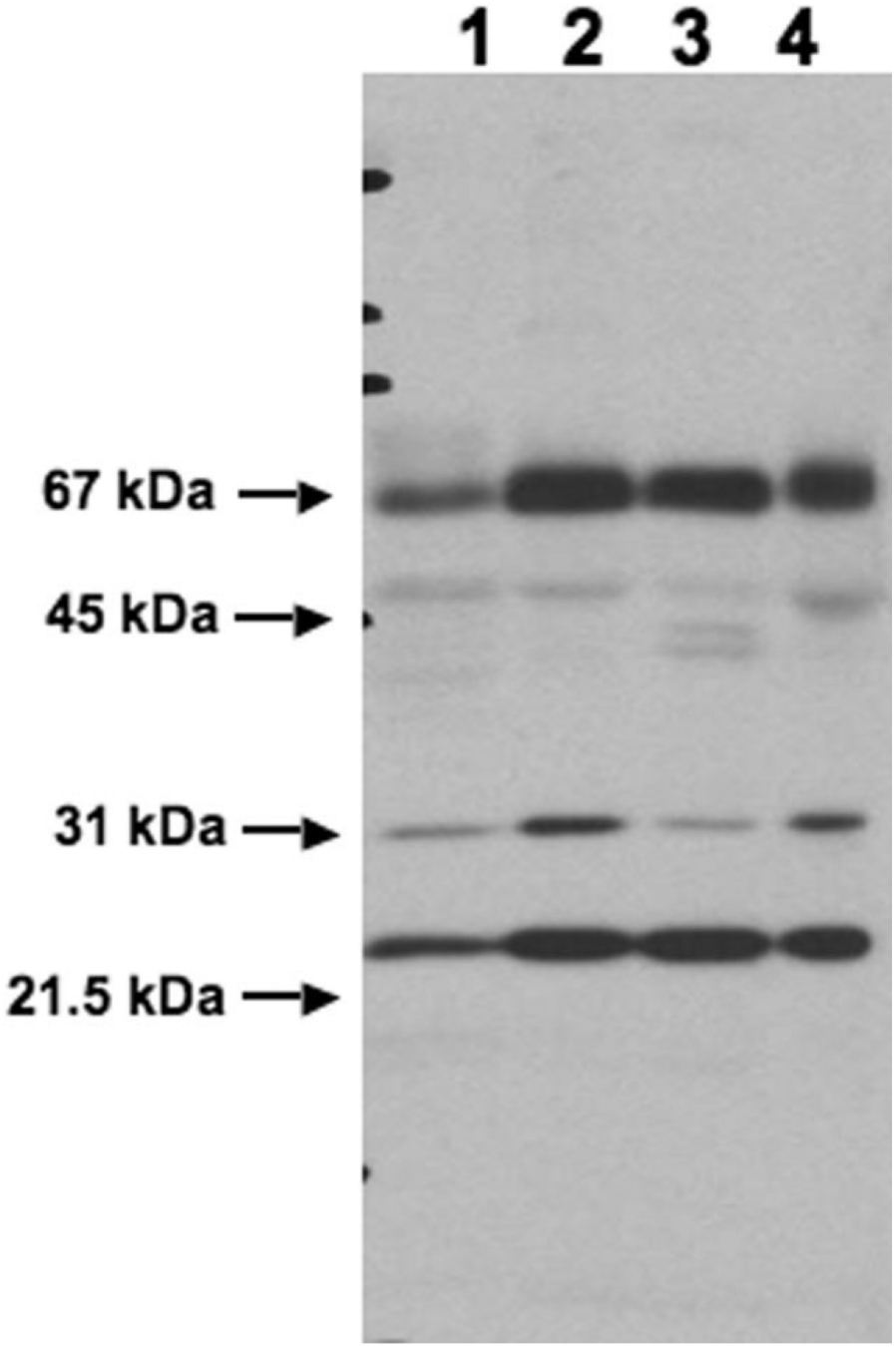

Fig. 6.

A representative immunoblot of anti-nGLT1 antibody in human cerebral white matter lysates. Lane 1 contains an adult human cerebral white matter standard made from three brains 55, 65, and 75 years old. Lanes 2 and 3 contain cerebral white matter (2) and cerebral gray matter (3) from the same 30-week-old preterm infant. Lane 4 contains another case of cerebral white matter from a 30-weekold preterm infant. The molecular weight of GLT1 is 67 kDa.

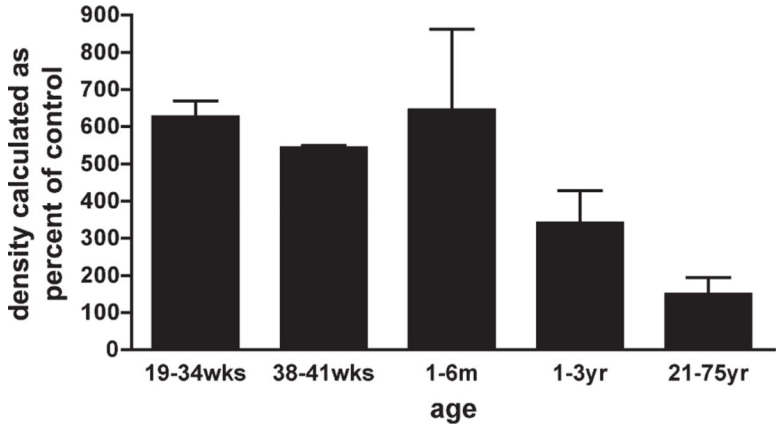

Fig. 7.

Comparison of protein expression of EAAT2 at different ages. Densitometric values were grouped as shown. A one-sample *t*-test was used to compare each sample group with adult standard (21–75 years), giving a significant *P* value of 0.001 for 19–41 weeks.

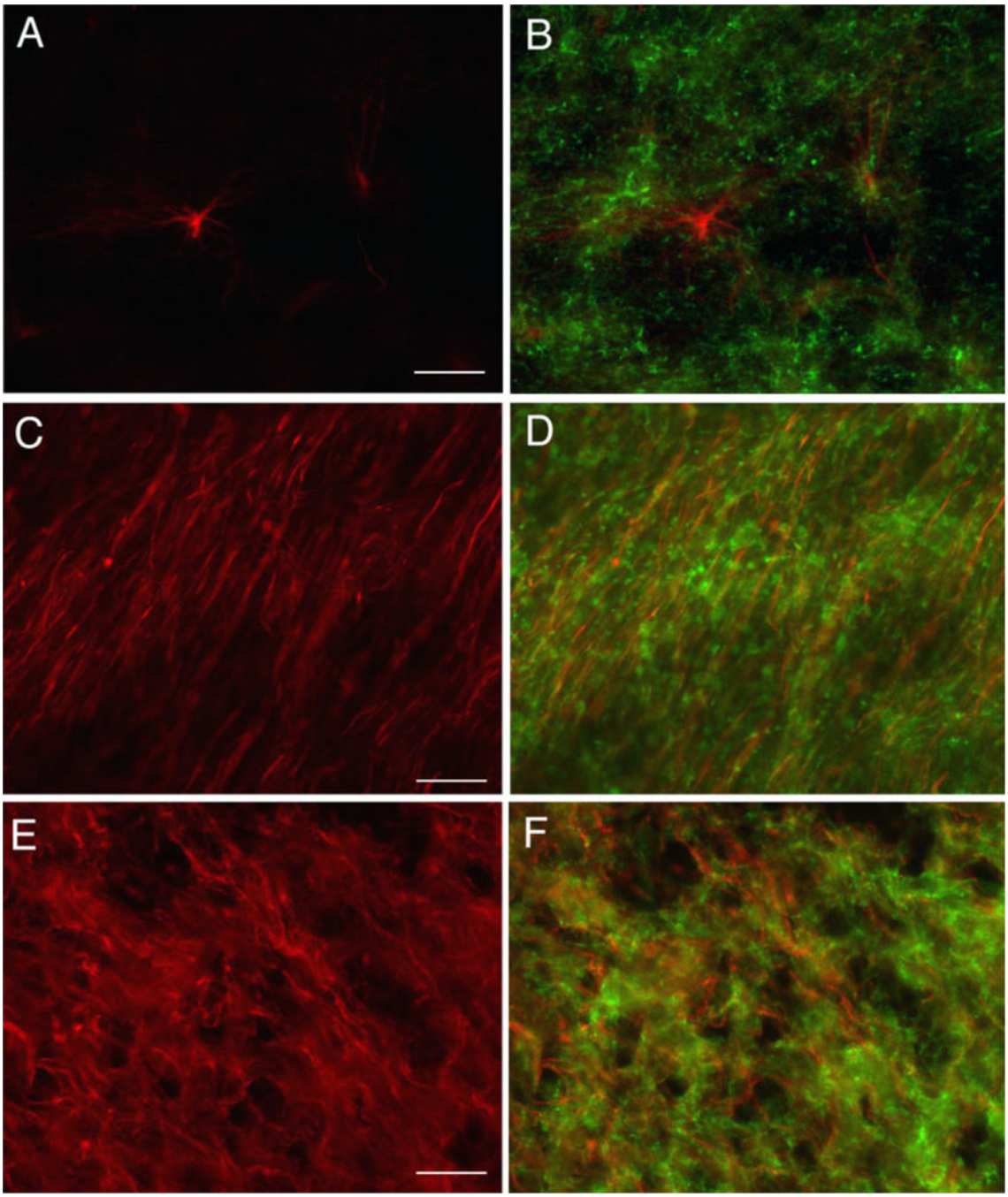

Fig. 8.

Immunocytochemistry of EAAT2 and cell-specific markers in human cerebral white matter. Fluorescence microscopy (×20) of 31-week-old human cerebral white matter labeled with GFAP (**A**), vimentin (**C**), and neurofilament (**E**). Double labeling of GFAP, vimentin, and neurofilament was performed with anti-GLT1 antibody, and the overlay shows no colocalization of GLT1 with these markers in **B**, **D**, and **F**, respectively. Scale bars = 50 μm.

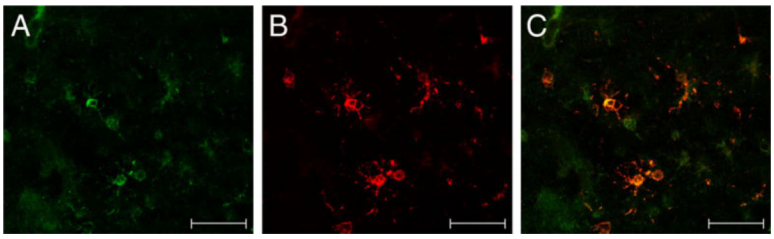

Fig. 9.

Immunofluorescent double labeling of EAAT2 and $O4^+$ OLs in human developing cerebral white matter. Fluorescence confocal microscopy (×40) of cerebral white matter from a 31-week-old preterm infant labeled with anti-nGLT1 polyclonal antibody (**A**) counterstained with the O4 monoclonal antibody (**B**). Overlay of A and B shows EAAT2 labeling in $O4^+$ OLs (**C**). Scale bars = 50 μm.

TABLE 1.

Summary of Human Cases Used for In Situ Hyridization, Immunoblotting, and Immunocytochemistry[1]

Case No.	Age	PMI	In situ	Blot	ICC
1	19 Weeks	4 Hours		×	
2	20 Weeks	4 Hours		×	
3	21 Weeks	24 Hours		×	
4	22 Weeks	4 Hours		×	
5	23 Weeks	4 Hours	×	×	×
6	23 Weeks	Uk			
7	24 Weeks	26 Hours		×	
8	25 Weeks	Uk	×		
9	26 Weeks	62 Hours		×	
10	30 Weeks	24 Hours		×	
11	31 Weeks	6 Hours			
12	31 Weeks	4 Hours		×	×
13	32–33 Weeks	22 Hours	×		×
14	34 Weeks	17 Hours	×		
15	34 Weeks	10 Hours		×	
5	38 Weeks	16.5 Hours	×		
6	38 Weeks	5 Hours	×	×	×
7	40 Weeks	24 Hours	×	×	
8	42 Weeks	41 Hours	×		
9	45 Weeks	7 Hours	×	×	
10	49 Weeks	12 Hours	×	×	
11	53 Weeks	12 Hours	×	×	
12	54 Weeks	16 Hours	×		
13	55 Weeks	Uk	×		
14	74 Weeks	20 Hours	×	×	
15	106 Weeks	21 Hours		×	
16	114 Weeks	27 Hours		×	
17	3 Years	Uk			
18	3 Years	20 Hours	×		
19	3.3 Years	Uk			×
20	21 Years	13 Hours	×	×	
21	38 Years	5 Years		×	×
22	43 Years	21 Hours	×	×	
23	61 Years	Uk	×		

[1]Age, gestational + postconceptional age; PMI, post-mortem interval; Uk, unknown.

TABLE 2.

Antibodies Used For Single- and Double-Label Immunocytochemistry and Western Blot[1]

Antibody	Source	Host and type	Antigen	Specificity	Dilution
Neurofilament (SMI312)	Sternberger Monoclonals, Lutherville, MD	Mouse mono IgG1 (mixture)	Homogenized hypothalami from Fisher 344 rats	Detects neruofilaments on axons in human fetal brain[2]	1:500
Vimentin (MAB 3400)	Chemicon International	Mouse mono IgG	Purified porcine vimentin	Detects a 57-kDa band specific for vimentin on immunoblots of human glioma cell lines[3]	1:1,000
GFAP (SMI 22)	Sternberger Monoclonals, Lutherville, MD	Mouse mono IgG (mixture)	Purified bovine GFAP	All components (clones 1B4, 2E1, 4A11) are specific for GFAP by radioimmunoassay, Western blot and immunocytochemistry[4]	1:500
O1	Gift from Dr S. Pfeiffer, Farmington, CT	Mouse mono IgM	Bovine corpus callosum	Recognizes specifically GalC and MG expressed on OL surface on lipid immunodot blots[5,6]	1:500
O4	Gift from Dr. S. Pfeiffer	Mouse mono IgM	Bovine corpus callosum	Reacts selectively with OL surface antigens sulfatide and seminolipid on lipid immunodot blots[5,6]	1:500
MBP (SMI 99)	Sternberger Monoclonals	Mouse mono IgG	Human MBP peptide containing aa 131-136 (–A–S–D–Y–K–S–)	Detects four bands between 14 and 21 kDa, corresponding to four MBP isoforms on immunoblots of rat cerebellum[7]	1:1,000
NeuN (MAB 377)	Chemicon International	Mouse mono IgG	Purified cell nuclei from mouse brain	Recognizes two bands on Western blot of mouse brain, corresponding to the 46- and 48-kDa NeuN isoforms[8]	1:100
nGLT1	Rosenberg Lab	Rabbit polyIgG	Rat peptide containing aa 1-15 (M–A–S–T–E–G–A–N–N–M–P–K–Q–V–E)	Recognizes one band on immunoblot of rat brain (67 kDa); does not stain GLT1 knockout using immunocytochemistry and immunoblotting[9,10]	1:500

[1] Poly, polyclonal; mono, monoclonal; aa, amino acids; GFAP, glial fibrillary acidic protein; O4, O1, OL progenitor surface markers; MBP, myelin basic protein; NeuN, neuronal nuclei; nGLT1, rat glutamate transporter homologue/EAAT2 in human.

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