Functional Glutamate Transport in Rodent Optic Nerve Axons and Glia

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KEY WORDS
axon; glia; glutamate transport

ABSTRACT
Recent findings suggest that synaptic-type glutamate signaling operates between axons and their supporting glial cells. Glutamate reuptake will be a necessary component of such a system. Evidence for glutamate-mediated damage of oligodendroglia somata and processes in white matter suggests that glutamate regulation in white matter structures is also of clinical importance. The expression of glutamate transporters was examined in postnatal Day 14–17 (P14–17) mouse and in mature mouse and rat optic nerve using immuno-histochemistry and immuno-electron microscopy. EAAC1 was the major glutamate transporter detected in oligodendroglia cell membranes in both developing and mature optic nerve, while GLT-1 was the most heavily expressed transporter in the membranes of astrocytes. Both EAAC1 and GLAST were also seen in adult astrocytes, but there was little membrane expression of either at P14–17. GLAST, EAAC1, and GLT-1 were expressed in P14–17 axons with marked GLT-1 expression in the axolemma, while in mature axons EAAC1 was abundant at the node of Ranvier. Functional glutamate transport was probed in P14–17 mouse optic nerve revealing Na+-dependent, TBOA-blockable uptake of D-aspartate in astrocytes, axons, and oligodendrocytes. The data show that in addition to oligodendroglia and astrocytes, axons represent a potential source for extracellular glutamate in white matter during ischaemic conditions, and have the capacity for Na+-dependent glutamate uptake. The findings support the possibility of functional synaptic-type glutamate release from central axons, an event that will require axonal glutamate reuptake.

INTRODUCTION

High-affinity, Na+-dependent glutamate transporters are responsible for clearing synthetically released glutamate from the extracellular space of the CNS (Anderson and Swanson, 2000; Danbolt, 2001; Gegelashvili and Schousboe, 1998). Recent findings suggest that synaptic-type glutamate release is present in CNS white matter (Kukley et al., 2007; Ziskin et al., 2007). Effective glutamate reuptake mechanisms must exist in white matter if this is the case. Of the five glutamate transporters that have been cloned, three are widely distributed in the mature CNS (Danbolt, 2001; Furuta et al., 1997b; Gegelashvili and Schousboe, 1998; Rothstein et al., 1994).

Glutamate-aspartate transporter (GLAST or EAAT1) and glutamate transporter 1 (GLT-1 or EAAT2) are commonly known as glial glutamate transporters, whereas excitatory amino acid carrier 1 (EAAC1 or EAAT3) is regarded as the principal transporter in neurons.

In addition to the physiological importance of white matter glutamate reuptake, the voltage and Na+-dependence of these glutamate transporters can result in the reversals of transport under ischaemic conditions, which may act as one source of the extracellular glutamate that is responsible for excitotoxic cell injury during disorders such as stroke (Anderson and Swanson, 2000; Attwell et al., 1993). In addition to injury in gray matter regions of the brain, stroke affects white matter regions that are made up of axons, astrocytes, and oligodendrocytes (Stys, 2004). Excitotoxic injury has also been implicated in other forms of white matter disease such as spinal cord injury and multiple sclerosis (Li et al., 1999; Matute et al., 2001). Oligodendrocyte damage is central to loss of function in ischaemic white matter injury and is thought to result from glutamate-mediated excitotoxicity (Dewar et al., 2003; Pantoni et al., 1996; Tekkok and Goldberg, 2001).

In addition to adult disease, white matter injury is a particularly important component of the lesions that underlie neuro-developmental disorders such as cerebral palsy, which in many cases appear to be ischaemic in origin (Kinney and Back, 1998; Volpe, 2001, 2003). Ischaemia evokes significant rises in extracellular glutamate concentration in white matter (Chiu and Kriegler, 1994; Shimada et al., 1993), and recent immuno-electron microscopy studies suggest that glutamate is released from both astrocytes and axons in ischaemic developing white matter (Wilke et al., 2004). There is little information about the type of transporters mediating glutamate uptake in white matter tracts of the central nervous system. Prior studies have shown EAAC1, GLAST, and...
GLT-1 expression in mature white matter (Choi and Chiu, 1997; Domercq and Matute, 1999; Furuta et al., 1997b; Kugler and Beyer, 2003), although the cellular distribution of these transporters is controversial, and little is known regarding their cellular distribution in developing white matter. Several studies have shown GLT-1 expression in neonatal central axons using light microscopy (Furuta et al., 1997b), but the close apposition of axonal and glial membrane casts the axonal expression of this “glial” transporter into doubt. However, membrane expression of GLT-1 in embryonic spinal cord axons before the appearance of astrocytes has been shown using immuno-electron microscopy (Yamada et al., 1998). Recent studies have shown that interruption of glutamate transport in white matter leads to excitotoxic injury of axons and glia (Domercq et al., 2005), highlighting the importance of glutamate clearance in these structures.

Here we use transgenic mice, where EGFP expression is under the control of glial cell-type specific promoters, coupled with standard and electron immuno-histochemistry to examine glutamate transporter expression in the P14–17 and mature optic nerve. Staining for the uptake of exogenously perfused d-aspartate and for the localization of glutamate was used to confirm that transporter expression was functional, revealing high levels of transporter expression in glia and in particular within axons.

### MATERIALS AND METHODS

#### Ethical Approval

All animal procedures were approved by local ethical review and conformed to UK home office regulations.

#### General

Transgenic mice (FVB/N) carrying the EGFP coding sequence under the control of CNP promoters 1 and 2 (Yuan et al., 2002) were kindly donated by the laboratory of Vittorio Gallo (CNMC Research, Washington DC). Heterozygous males were mated with wild-type females and transgenic littermates identified. A tendency for epileptic seizures in the colony was largely removed by out-breeding with wild-type animals and subsequent back-crossing. Transgenic mice (FVB/N) with GFP under control of the GFAP promoter were obtained from The Jackson Laboratory (Bar Harbour, Maine). GLT-1 and GLAST knockout mice were a generous gift from David Attwell’s laboratory (University College London), together with wild-type littermates from the GLT-1 mice. All animals were maintained in accordance with local ethical guidelines.

#### Immuno-Histochemistry

For immuno-histochemistry, wild-type optic nerves from P14–17 mice were dissected in 0.1 M PBS and fixed in 4% paraformaldehyde for 30 min. A minimum of three nerves from three different animals were analyzed for each age/antibody. Nerves from >P30 animals are termed “adult” throughout. The optic nerves were subsequently incubated in 0.1 M PBS plus 20% sucrose w/v for 5 min prior to freeze-sectioning and subsequent blocking for 60 min in 0.1 M PBS 10% fetal goat serum plus 0.5% Triton-X 100 and incubated in this solution plus primary antibody at 4°C overnight. Affinity-purified rabbit polyclonal antibodies against GLT-1, GLAST, and EAAC1 together with specific blocking peptides were obtained from Alpha Diagnostics (San Antonio, TX) and were used at a 1:100 dilution. The amino acid sequences used for production of all the transporter antibodies used in this study are shown in Table 1 (GLT-1 (1) and GLAST (1) are the above-mentioned antibodies). Mouse monoclonal antibodies against neurofilament-70 (NF-70, 1:200) were obtained from Chemicon Europe (Southampton, UK). Affinity-purified rabbit polyclonal antibody raised against d-aspartate (1:2,000) was obtained from Cell Sciences (Canton, MA) and has a cross-reactivity ratio of 1:10,000 for L-aspartate determined by ELISA (information provided by Cell Science). Rabbit polyclonal anti CNPase was obtained from Sigma UK (1:100). The appropriate Alexa-conjugated secondary antibody (Cambridge Bioscience, UK) was applied for 120 min following washing, and single-plane fluorescent sections were imaged at ×60 on an Olympus scanning confocal microscope. Slow scanning and image averaging was required to resolve individual axons, which range from 0.1 to 1 µm in diameter at this age. The resulting degree of photo-bleaching confounded attempts to collect image stacks. Alexafluor-568 conjugated secondary antibodies were employed when labeling transgenic mice to allow spectra discrimination between the label and EGFP (emission maxima at 520 nm). In all cases, each staining protocol was performed on a minimum of three optic nerves from three separate animals.

The same protocol was used for immunohistochemistry of adult rat optic nerve except for the antibodies that were as follows: affinity-purified rabbit polyclonal antibodies to GLAST (0.1 µg/mL), GLT-1, and EAAC1 (both 1 µg/mL) (see Table 1 GLT-1 (2) and GLAST (2)).
Anti-GLAST antiserum was a generous gift by Dr. N. Danbolt, (Lehre et al., 1995), and anti-GLT-1 and anti-EAAC1 were from Dr. J. Rothstein (Rothstein et al., 1994). Mouse monoclonal antibody against Na⁺ channels present at the nodes of Ranvier (PAN K58/35, 1 μg/mL) was obtained from Sigma. The appropriate Alexa-conjugated secondary antibodies were used together with Hoechst 33258 (5 μg/mL, Sigma), which allows visualization of the cellular nuclei.

**d-Aspartate Uptake**

For d-aspartate uptake studies, optic nerves from wild type and transgenic mice were dissected and placed in artificial cerebrospinal fluid (aCSF), composition (in mM): NaCl, 126; KCl, 3; NaH₂PO₄, 2; MgSO₄, 2; CaCl₂, 2; NaHCO₃, 26; glucose, 10; pH, 7.45, bubbled with 5% CO₂/95% O₂ and maintained at 37°C. This solution contained 500 μM d-aspartate and trials indicated that a 60 min incubation period produced good cell loading. Shorter periods of incubation failed to produce reliable d-aspartate staining and the relatively prolonged incubation period presumably reflects the slow penetration of d-aspartate into this whole-mount preparation. A very similar protocol has been previously used in the isolated lamprey spinal cord, a preparation that is physically similar to the isolate rodent optic nerve (Gundersen et al., 1995). Given the relatively long incubation, it is possible that d-aspartate will be partially metabolized in the optic nerve, although the amino acid is thought to be largely nonmetabolizable (Bender et al., 1997). Following the incubation, optic nerves were washed for 5 min in aCSF and fixed in 3% glutaraldehyde prior to processing for immuno-histochemistry (see above). Zero-Na⁺ aCSF had NaCl replaced with N-methyl-D-glucamine, NaH₂PO₄ replaced with KH₂PO₄, KCl reduced to 1 mM and NaHCO₃ replaced with choline-HCO₃ (pH 7.45). All chemicals were obtained from Sigma (UK).

**Electron Microscopic Immuno-Histochemistry**

For pre-embedded glutamate transporter electron microscopic immuno-histochemistry in adult white matter, we used five Sprague–Dawley rats. Deeply anesthetized animals were transcardially perfused with 500 mL of fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). Both longitudinal and transversal vibratome floating sections were preincubated in 10% goat serum in PBS (blocking solution) for 1 h. Sections were then incubated with the anti-GLAST (5 μg/mL), anti-GLT-1 (0.4 μg/mL), or anti-EAAC1 (1.14 μg/mL) antisera diluted in blocking solution for 3 days at 4°C with continuous shaking. Antibodies are listed in Table 1 for use with rats (marked “2”). Since the epitopes targeted in these experiments are intracellular, the antibodies are likely to stain only transected cells. In negative control sections, the primary antibody was omitted. After several washes in PBS, tissue sections were incubated in 1.4 nm gold-labeled goat anti-rabbit IgG (Fab’ fragment, 1:100 in blocking solution; Nanoprobes) for 4 h. Following washes, gold particles were silver-intensified with an HQ Silver Enhancement kit (Nanoprobes) for about 10 min. Tissue sections were postfixed with 1% osmium tetroxide, dehydrated, and embedded in epoxy. Ultrathin sections were counter-stained with uranyl acetate and lead citrate and examined using a PHILIPS EM208S electron microscope. To estimate the density of silver-intensified particles present in each cellular element (astrocytes, oligodendrocytes, myelin, axon tracts, and nodes of Ranvier), electron microphotographs were taken. Scion Image software (NIH, Frederick, MD) was used to measure the area and the number of immunoparticles within cells (excluding those in mitochondria and nuclei). For membrane counts, only gold particles within 30 nm of the membrane were included. Particles located within mitochondria and/or nucleus were assumed to reflect the level of background staining and the density of staining in these structures was subtracted from the membrane counts.

For postembedded glutamate, glutamate transporter and d-aspartate (nerves incubated at 500 μM for 60 min) electron microscopic immuno-histochemistry optic nerves from P14–17 wild-type mice were postfixed in 3% glutaraldehyde/Sorenson’s buffer. Nerves were postfixed with 2% osmium tetroxide and dehydrated prior to infiltration in epoxy. Sections were counterstained with uranyl acetate and lead citrate and examined with a Jeol 100CX electron microscope, see (Thomas et al., 2004) for further details. Antibodies and concentrations were the same as listed above for mice light immuno-histochemistry at this age (marked “1” in Table 1), with the exception that the anti-d-aspartate was used at a concentration of 1:1,000. Secondary antibodies were goat anti-rabbit conjugated to 30-nm gold particles (British Biocell).

**Cell Type Identification**

Astrocytes in the developing optic nerve can vary widely in somata size and may not contain the typical glial filaments, but can generally be identified by the presence of a wide-bore endoplasmic reticulum, glycogen granules, a relatively dark cytoplasm, and an irregular-shaped nucleus with chromatim clumped under the envelope. Astrocytes also extend processes to form the glial limitans and the glial end feet of capillaries. Oligodendroglia generally have larger somata and may have myelinating axons embedded in the somata or processes. Other features used to identify cells of this lineage include a relatively light cytoplasm, numerous large mitochondria, often at one pole of the somata, large nuclei of an oval shape containing evenly dispersed chromatim, the presence of two nucleoli, narrow bore endoplasmic reticulum, and the absence of astrocyte features such as glycogen granules. Axons can be reliably identified by the presence of neurofilaments and microtubules. For further details and examples of cell identification in...
developing optic nerve using similar fixing and staining protocols, see Wilke et al. (2004) and Thomas et al. (2004).

### Statistics

Statistical differences were assessed by Student’s *t*-test or ANOVA test as appropriate. Particle count data are presented as mean ± SEM.

### RESULTS

**High Affinity Glutamate Transport Expression in P14–17 Mouse Optic Nerve**

EAAC1, GLAST, and GLT-1 were all detected in wild-type P14–17 optic nerve by antibody staining (Fig. 1, left panels). In each case, the staining was ablated by incubation with a specific blocking peptide (Fig. 1, right panels). Antibody specificity was further checked by performing staining on brain sections from P14–17 GLAST and GLT-1 knockout mice (see Fig. 2). Two different anti-GLT-1 antibodies are used for different protocols in this study (see Table 1) and no detectible reactivity was seen for either in the GLT-1 knockout animals, while cell staining was apparent in both wild-type littermates (to the GLT-1 mice) and in the GLAST knockouts (Fig. 2, top two rows). In contrast, detectible GLAST staining was not found in the brains of GLAST knockout animals, but was seen in the GLT-1 knockouts and the wild-type controls (Fig. 2, bottom row). In wild-type optic nerve, EAAC1, GLAST, and GLT-1 staining was apparent in numerous cell bodies (Fig. 1, arrows). Staining of somata will correspond to glial cell bodies in this neuron somata-free preparation. Expression of all three proteins was not restricted to somata and staining was seen throughout the nerve presumably due to staining of glial processes and/or axons. Such staining is similar to that described previously (Li et al., 1999), and was at a somewhat lower level of intensity to that seen in the somata. In the current investigation, we initially imaged control sections to determine the imaging parameters required to eliminate background fluorescence prior to using the same parameters for tests sections, thus ensuring that our images were not biased toward bright objects at the expense of dim objects.

**D-Aspartate Uptake Via High Affinity Glutamate Transport in P14–17 Mouse Optic Nerve**

Functional high affinity Na⁺-dependent glutamate transporters will transport exogenous d-aspartate into cells that can subsequently be assessed by antibody staining (Gundersen et al., 1993, 1995). Perfusion with 500 μM d-aspartate for 60 min produced extensive d-aspartate staining in somata and non-somatal regions of the P14–

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**Fig. 1.** Glutamate transporter expression in P14–17 optic nerve. EAAC1 (a), GLAST (b), and GLT-1 (c) expression has both a somata pattern of expression (arrows) and non-somata pattern of expression in P14–17 optic nerve. Antibody staining is not seen when the antibodies are incubated in the relevant blocking peptides (right side). Scale bar = 10 μm.

**Fig. 2.** The specificity of the GLT-1 and GLAST antibodies. Immunostaining using the GLT-1 antibody used for light microscopy and post-embedded immuno-electron microscopy “GLT-1 (1),” and that used for pre-embedded immuno-electron microscopy “GLT-1 (2)” and for the GLAST antibody. Staining was performed on mice brain sections from GLAST knockout animals, GLT-1 knockout animals, and wild-type littermates of the GLT-1 knockout. Note that no GLT-1 immuno-reactivity was detected using either GLT-1 antibody in the GLT-1 knockout brain and no GLAST reactivity was detected in the GLAST knockout brain. Scale bar = 20 μm.
GLUTAMATE TRANSPORTERS IN GLIA AND AXONS

17 optic nerve (Fig. 3a). Preabsorbing the D-aspartate antibody for 30 min in 500 μM D-aspartate prior to staining ablated the immuno-staining (Fig. 3b). D-Aspartate uptake was largely abolished by zero-Na\(^+\) conditions (Fig. 3c) and by the specific transport inhibitor D-threo-β-benzylxoxaspartate (TBOA, 10 μM; Fig. 3d), confirming the expression of functional high affinity Na\(^+\)-dependent glutamate transport in somata and non-somatal regions of the optic nerve. This relatively low TBOA concentration was used to minimize hetero-exchange of TBOA for intracellular D-aspartate (Anderson and Swanson, 2000; Montiel et al., 2005), but will result in only partial inhibition of GLAST. This may explain why D-aspartate staining appears to be lower under zero-Na\(^+\) conditions, which will block all the glutamate transporters.

Functional Glutamate Transporter Expression in Axons in P14–17 Mouse Optic Nerve

The expression pattern of GLT-1, GLAST, and EAAC1 and the pattern of uptake of D-aspartate into areas surrounding somata may indicate axonal expression of glutamate transporters in developing optic nerve. Double labeling for neurofilament suggested co-localization of this axonal marker with GLT-1, EAAC1, and GLAST (Fig. 4a–c). Immuno-gold labeling for EAAC1 revealed that while this transporter is expressed within oligodendrocyte processes that are closely apposed to axons, and to a limited extent within the axoplasm, no staining was apparent within the axolemma itself (Fig. 4d,e). Postembedded immuno-staining for GLAST was not successful, but immuno-gold GLT-1 labeling revealed extensive expression within the axolemma both of premyelinated and myelinated axons, in addition to staining of near-by astrocyte processes (Fig. 4f,g).

Light microscopy suggested that D-aspartate uptake occurs into axons within nerves previously incubated with this amino acid (Fig. 5a,b, arrows), in addition to uptake into glial processes running parallel to axons (Fig. 5c,d, arrows). Although axonal D-aspartate uptake was somewhat punctate in many regions, areas of diffuse staining along the center of axons were apparent and presumably represent axoplasmic accumulation. At the ultrastructural level, postembedded immuno-gold labeling for glutamate in control nerves showed the presence of the neurotransmitter in astrocytes, axons, and oligodendrocytes (Fig. 6a). The presence of D-aspartate in incubated nerves confirmed the loading of the amino acid into axons and oligodendrocytes, with highly variable loading into astrocytes (Fig. 6b). No staining was apparent in control nerves (Fig. 6c). Postembedded staining was used in the P14–17 tissue since in our hands it allows a greater level of tissue detail to be observed, which is required to examine subcellular expression in small diameter P14–17 axons.

Blinded counting of gold particles showed that astrocytes accumulate significantly more D-aspartate than either axons or oligodendrocytes within incubated optic nerves, although between them axons and oligodendrocytes have a greater total capacity for D-aspartate uptake (axon + oligodendrocyte uptake) than do astrocyte alone (Fig. 6d). The population distribution of gold-particle density within astrocytes was found to be highly variable, with a population of astrocytes that accumulated little D-aspartate and other cells that accumulated much more (Fig. 6e). Particle counts were collected from 2 optic nerves from separate animals, with 16 astrocytes, oligodendrocytes, and axons analyzed from a total of 8 sections (2 of each from each section).

Functional Glutamate Transporter Expression in Astrocytes in P14–17 Mouse Optic Nerve

Transgenic mice, in which EGFP expression is under the control of glial cell-specific promoters, were used to probe the expression of glutamate transporters and the uptake of D-aspartate into astrocytes and oligodendrocytes. Optic nerves from P14–17 EGFP-GFAP mice retained EGFP fluorescence following fixation, although not all astrocytes in this mutant express detectable levels of fluorescent protein (Nolte et al., 2001; Zhuo et al., 1997). Immuno-staining revealed an absence of EAAC1, and a relatively high level of GLT-1 expression in these cells (Fig. 7a,b). Only diffuse staining for GLAST was found in identified glial cells in P14–17 mouse optic nerve, which could not be discriminated from background levels of staining (not shown). The occasional GLAST (+) soma seen in Fig. 1b are presumably therefore neither astrocytes nor oligodendrocytes, and may possibly be precursor cells. At the ultrastructural level, gold particles for GLT-1 were present at high den-
Fig. 4. Glutamate transporter expression in P14–17 mouse optic nerve axons. (a–c) Axons are co-stained with NF-70 (left panels, green) and for either: EAAC1 (a, middle panel, red), GLAST (b, middle panel, red), or GLT-1 (c, middle panel, red). The images are overlaid on the panels to the right, showing what appears to be co-localization of all three transporters in axons (yellow). (d, e) Immuno-gold reactivity for EAAC1. (d) Gold particles are found throughout the cytoplasm and in particular in the cell membrane of oligodendrocytes (arrows). The area in the white box is shown in greater detail in the black box. (e) Staining is evident in oligodendrocyte processes (asterisks + arrows) that are closely apposed to axons (Ax). Light staining was found in the axoplasm of axons (arrow head); but not in the axolemma. Note on the right a rare gold particle in the cell membrane of an astrocyte process (ap) aligned next to an axon (arrow). (f, g) Immuno-gold reactivity for GLT-1. Note the heavy gold-particle labeling within the axolemma in both panels (arrows), in addition to staining within the axoplasm and within the membrane of astrocyte processes (ap) (arrows). The areas within the white boxes are shown at higher gain below. Scale bar = 10 μm in (a–c) and 1 μm in (d–g). The cell types are identified using standard criteria for this preparation (Thomas et al., 2004).
Assessed at the light microscopic level, the capacity of astrocytes to take up exogenous D-aspartate was found to be highly variable with some cells having undetectable levels of D-aspartate and others having very high levels (Fig. 7d,e). This tendency was not related to the position of the cells within the nerve and was unlikely to arise from preferential loading of D-aspartate into more peripherally located astrocytes.

**Functional Glutamate Transporter Expression in Oligodendroglia in P14–17 Mouse Optic Nerve**

At the light microscopic level, the majority of oligodendrocytes in P14–17 EGFP-CNP optic nerve expressed EAAC1, with only diffuse low levels of staining observed for GLT-1 (Fig. 8a–d). A population of EAAC1 (−) oligodendrocytes were also observed (not shown). D-Aspartate uptake into oligodendrocytes was at a somewhat higher level than that seen in surrounding tissue (Fig. 8e–h) and D-aspartate (−) cells were not seen. Uptake was often particularly marked within processes of EGFP-CNP oligodendrocytes (Fig. 8g), but the EGFP fluorescence was retained only poorly in the more distal processes that are beginning to ensheath axons at this point in development. In separate experiments, oligodendrocytes in preloaded wild-type mice were antibody-stained against CNPase to show these fine processes in greater detail, revealing D-aspartate uptake into these structures (Fig. 8e,f, arrows). This may explain how D-aspartate is present in the EAAC1 (−) somata, if the amino acid is taken up in the processes before diffusing throughout the cytoplasm. Alternatively, D-aspartate into these somata may result from some form of low affinity uptake. This series of stainings also revealed CNPase (−) axon-like structures loaded with D-aspartate (Fig. 8e,f, arrow heads), which correspond to the NF-70 (+) axons seen in Fig. 5.

**Glutamate Transporter Expression in Mature Optic Nerve**

Glutamate transporter expression was also examined in mature mouse and rat optic nerve by electron microscopy, revealing a similar pattern in both (we will show the data from the rat). Mature optic nerve astrocytes were found to mainly express GLT-1 in their somata and processes (Fig. 9a), in agreement with the idea that this is the major glutamate transporter present in these cells. GLT-1 immuno-particles are almost exclusively found in plasma membranes within astroglia. As in P14–17 optic nerves, GLT-1 was also expressed in oligodendrocytes but in the adult cells it was found mostly in plasma membranes (Fig. 9b). No GLT-1 expression was found in axonal tracts of the adult optic nerve (Fig. 9a,c,d).

GLAST was also expressed in both astroglial somata and processes of the adult optic nerve (see Fig. 10), but the density of immuno-particles was lower than for GLT-1. As for GLT-1, GLAST was mostly associated with plasma membranes of astrocytes. GLAST was also found in oligodendrocytes, especially in their myelin sheaths (Fig. 10a,c, arrows). GLAST was not detected in axonal tracts including the nodes of Ranvier (Fig. 10c,d).

Most oligodendrocytes and astrocytes in the adult optic nerve expressed EAAC1 (see Fig. 11). In addition, EAAC1-immunolabeling was found in axons and at the nodes of Ranvier (Fig. 11a,c,d). Particles were located in the axoplasm of nodes and also at the nodal membrane; however, in internodal regions there was scattered immuno-labeling. EAAC1 was also present in the cytoplasm of astrocytes and oligodendrocytes in the optic nerve, a feature which could be due to the rapid trafficking and recycling of this transporter between the cytoplasm and the plasma membrane (Danbolt, 2001).
The expression of glutamate transporters at the nodes of Ranvier in adult rat optic nerve was also examined by immunofluorescence using an antibody against Na\(^+\) channels, a marker of this structure. Consistent with our EM findings, we observed the presence of EAAC1, but not of GLT1 and GLAST in this structure (see Fig. 12).

A quantitative analysis of the distribution of glutamate transporters revealed that GLT-1, GLAST, and
EAAC1 are all expressed at high levels in astrocytes, with GLT-1 the most heavily expressed (Fig. 13a). In addition, EAAC1 was abundant in oligodendrocyte somata and GLT-1 was present in these cells to a lesser degree, with GLAST present in the myelin sheath (Fig. 13a). EAAC1 was found at high levels in axons, particularly, at the nodes of Ranvier. Particles were counted in 5 optic nerves from separate animals with a total of 482 astrocytes, 183 oligodendrocytes, 786 myelin sheaths, 173 nodes of Ranvier, and 800 axonal tracts analyzed. The density of gold particles present in cell membranes was also assessed (Fig. 13b), which correlated well with the whole cell counts. Membrane counts were performed on 3 optic nerves from different animals with 156 astrocytes, 76 oligodendrocytes, and 87 nodes of Ranvier analyzed.

**DISCUSSION**

We have shown a high capacity for Na\(^+\)-dependent, TBOA-blockable, \(\nu\)-aspartate uptake into P14–17 mouse optic nerve astrocytes, axons, and oligodendrocytes and have documented high-affinity glutamate transporter expression in white matter of two rodent species at two developmental points. Axons, astrocytes, and oligodendroglia are also shown to contain cytoplasmic glutamate, a prerequisite of ongoing glutamate uptake although other sources of cytoplasmic glutamate exist. These findings indicate that glutamate regulation is important in central white matter tracts such as the optic nerve and that it is achieved by glutamate transporter expression in axons and oligodendroglia in addition to astrocytes. Glutamate transporters were expressed in optic nerve oligodendrocytes, axons, and astrocytes both during development and in the adult. This implies that the extracellular concentration of glutamate is carefully regulated by these proteins in white matter, just as it is in gray matter.

The current preparation, the rodent optic nerve, has the advantage that glial and axon maturation is relatively coherent and has been well characterized. For example, in the P14–17 rodent optic nerve ~10–25% of axons have received at least a first layer of myelin, with 10–15% of axons being contacted by oligodendrocyte processes but not yet receiving a myelin layer and ~65–80% of axons being premyelinated (all axons are myelinated in the adult) (Hildebrand and Waxman, 1984). The P14–17 rodent optic nerve is, therefore, at a similar developmental point to the term central white matter that is most commonly subject to hypoxic-ischaemic injury at birth (Brody et al., 1987; Craig et al., 2003; Inder and...
and GLT-1 expression in oligodendrocytes and axons. Kugler and Beyer (2003) used β-tubulin as an oligoden-
drocyte marker, which may also be expressed in astro-
cytes, for example, (Medrano and Steward, 2001), and it
is possible that some cells identified in their study as oli-
godendrocytes were in fact astrocytes. In addition to
optic nerve, high levels of GLAST, GLT-1, and EAAC1
expression have been reported in other white matter
structures such as subcortical white matter, in particu-
lar during postnatal development (Furuta et al., 1997b).

The current study used pre-embedded immuno-elec-
tron microscopy to demonstrate GLT-1, GLAST, and
EAAC1 expression within the cell membrane of mature
astrocytes and EAAC1 expression within the cell mem-
brane of oligodendrocytes. There has been no previous
examination of the cellular distribution of glutamate
transporter expression in developing white matter. The
current study employed EGFP expression linked to cell-
specific promoters to identify glial cell types in P14–17
mouse white matter, removing any possibility of anti-
body interactions during double labeling, and used post-
embedded immuno-electron microscopy to examine the
subcellular distribution of the proteins. At P14–17,
membrane expression of GLT-1 was found in astrocyte
processes, with only cytoplasmic expression seen in oli-
godendrocytes. EAAC1 was expressed in oligodendro-
cytes and not in astrocytes, while staining for glial
GLAST expression was not easily discriminated from
background levels. The major findings are summarized
in Figure 14. The EGFP-expression approach was also
used to examine mature mouse optic nerve, producing
results similar to those described with pre-embedded
immuno-electron microscopy. Uptake of exogenous D-
aspartate was also employed to prove that developing
optic nerve glial glutamate transporters were functional
in situ.

**Glutamate Transporter Expression in Axons**

The current study revealed intense membrane expres-
sion of GLT-1 in axons in P14–17 optic nerve. In con-
trast, mature axons did not express GLT-1 or GLAST
while strong EAAC1 expression was found largely local-
ized to the node of Ranvier. Previous studies have shown
GLT-1 expression in immature mouse spinal cord axons
up to the first postnatal week (Yamada et al., 1998), and
in fetal ovine central axons (Northington et al., 1998,
1999), while EAAC1 is present in mature cerebellar Pur-
kirje cell axons (Furuta et al., 1997a). While neuronal
GLT-1 mRNA has been reported in several studies
(Berger and Hediger, 1998; Schmitt et al., 1996), GLT-1
protein expression is generally absent from mature neu-
rions (see Danbolt, 2001; Rothstein et al., 1994), although
expression has been reported in axon terminals of hippo-
campal neurons (Chen et al., 2002, 2004; Schmitt et al.,
2002). Both GLT-1 and GLAST are expressed within the
retina (Euler and Wasse, 1995; Rauen and Kanner,
1994; Rauen et al., 1996), although expression of neither
protein has been found in the developing retinal gan-

**Glutamate Transporter Expression in White Matter**

White matter glutamate transporter expression has
previously been examined mainly in the adult rat optic
nerve, a preparation that contains protein and mRNA
for GLT-1, EAAC1, and GLAST (Choi and Chiu, 1997;
Domercq et al., 1999). Two groups have published differ-
tent cell distributions of these transporters in this prepa-
ration. Domercq et al. (1999) reported GLAST expression
in interfascicular oligodendrocytes, EAAC1 in a popula-
tion of unidentified glia and GLT-1 in astrocytes. In con-
trast, Kugler and Beyer (2003) found GLAST expression
in most astrocytes, a population of EAAC1 (+) astrocytes

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**Fig. 8. Glutamate transporter expression in P14–17 optic nerve oli-
godendrocytes. (a) GFP expression in a CNP-GFP oligodendrocyte
showing soma and processes. (b) EAAC1 reactivity of the same section.
Overlay on the right, showing EAAC1 expression on the soma and proc-
esses. (c) A single CNP-GFP (+) oligodendrocyte. (d) GLT-1 reactivity
of the same section. Overlay on the right, showing diffuse GLT staining
in the cell. (e) D-aspartate staining of a wild-type, D-aspartate incubated
nerve. (f) CNPase staining of the same section. Overlay below, showing
clear D-aspartate staining in the CNPase (+) oligodendrocyte processes
(small arrows) as well as within CNPase (−) axon-like structures
(arrow heads). (g) Three GFP (+) oligodendrocytes in a GFP-CNP
nerve. (h) D-aspartate staining of the same section. Overlay below,
showing D-aspartate staining within somata and proximal processes of
oligodendrocytes. Scale bars = 10 μm.**
GLUTAMATE TRANSPORTERS IN GLIA AND AXONS

Fig. 9. Ultrastructural localization of GLT-1 in adult rat optic nerve. (a) Astrocyte processes (ap) with intense GLT-1 immuno-labeling surrounding a group of axons (ax) all devoid of reactivity. (b, c) sections showing that GLT-1 is present at high levels in astrocyte processes (ap) and to a lesser extent in oligodendrocytes (oli), but not in axons (ax). (d) No expression of GLT-1 is observed in the internodal segment of axons (ax) and at the nodes of Ranvier (R). Scale bars = 300 nm. ast = astrocyte nucleus. Arrowheads point to examples of gold particles in plasma membranes.

Fig. 10. Ultrastructural localization of GLAST in adult rat optic nerve. (a, c) Transverse and longitudinal sections showing GLAST localization in astrocyte processes (ap) that surround some axons (ax) free of labeling. Arrows indicate the presence of immuno-gold particles in myelin sheaths. (b) Expression of GLAST in astrocyte processes and myelin sheaths (arrows). (d) Axons (ax) and the nodes of Ranvier (R) are unlabeled. Scale bars = 300 nm. Arrowheads point to examples of gold particles in plasma membranes.
Glial cells that extend the optic nerve axons (Reye et al., 2002). The high level of n-aspartate uptake and the localization of glutamate within axons confirmed the presence of functional transport, which in terms of n-aspartate uptake capacity was comparable to that seen in glial cells and occupied a larger proportion of the nerve. Axonal uptake was blocked by either Na\(^{+}\) removal or by the specific transport inhibitor TBOA, and this is the first direct proof of functional glutamate transport expression in axons. The high level of expres-

**Fig. 11.** Ultrastructural localization of EAAC1 in adult rat optic nerve. (a, c) Transverse and longitudinal sections showing EAAC1 expression in both axons (ax) and astrocyte processes (ap). (b) EAAC1 is also found in oligodendrocytes (oli) as well as in astrocyte processes (ap) in their vicinity. (d) EAAC1 reactivity is also found at the nodes of Ranvier (R). Scale bars = 300 nm. Arrowheads point to examples of gold particles in plasma membranes.

**Fig. 12.** Glutamate transporter expression at the nodes of Ranvier in adult optic nerve. Nodes of Ranvier are stained with PAN (green) and the same section is co-stained for either GLT-1, GLAST, or EAAC1 (red). Cell nuclei are stained with Hoechst in blue. The images are overlaid showing co-localization only for EAAC1 at the nodes (yellow). Transects of colocalization are shown. Scale bar = 20 \(\mu\)m.
The expression of EAAC1 at the nodes of Ranvier indicates a high level of glutamate regulation in the node in mature axons. EAAC1 immuno-particles are often present in the cytoplasm, a feature which could be interpreted as protein in transit to the plasma membrane (Danbolt, 2001).

Glutamate Transporters and White Matter Injury

At P14–17, oligodendrocytes accumulated high levels of D-aspartate during exogenous perfusion, which is consistent with the elevated levels of glutamate transporter expression in these cells at this point in development, in particular, EAAC1. Average D-aspartate uptake into astrocytes was higher than into any other cell compartment, although the degree of uptake varied considerably from cell to cell. It is unclear why some astrocytes accumulated low levels of D-aspartate, a phenomenon that did not appear to relate to cell position in the nerve or the presence of a population of astrocytes with low glutamate transport levels, which were not seen. One possibility is that some of the astrocytes are not yet physiologically competent, since it is known that many optic nerve astrocytes lack the morphological features of mature astrocytes at this age (Skoff et al., 1976). Recent studies have shown rapid astrocyte swelling leading to necrosis in ischaemic developing white matter, which will liberate astrocyte glutamate into the extracellular space in a glutamate transporter-independent fashion (Thomas et al., 2004). In addition, the presence of functional GLT-1 demonstrates the potential for transport-mediated glutamate release from these cells. Thomas et al. (2004) also demonstrated a high resting intracellular glutamate levels in oligodendrocytes that was elevated following ischaemia, suggesting both effective glutamate uptake under normal conditions and the absence of uptake reversal during ischaemia, although ischaemic glutamate release from these cells is also possible (Fern, 2000; Back et al., 2006). It is not known how [Na⁺], pHᵢ, and membrane potential are affected by ischaemia in these cells, but it would appear that the conditions are not met for significant reversal of glutamate uptake in this preparation (but see Back et al., 2006). The current findings suggest that glutamate release from axons via reverse transport is a potentially important factor in excitotoxic (Dewar et al., 2003; Wilke et al., 2004) and nonexcitotoxic (Oka et al., 1993) white matter damage during ischaemia.

White Matter Glutamate Transporter Function

Glutamate receptors are expressed in developing glia, and may regulate cell development and fate (Belachew and Gallo, 2004). Expression of AMPA/kainate receptors is particularly marked in immature cells of the oligodendrocyte lineage (Itoh et al., 2002), which in the de-
veloping optic nerve are in the process of forming an exquisitely close morphological arrangement with neighboring axons (Butt and Ransom, 1993). We and others have recently demonstrated the presence of functional NMDA-type glutamate receptors in oligodendrocyte processes (Kárádóttir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). The activation of these receptors is likely to be important in a variety of diseases and must have significant developmental consequences.

We have now shown high levels of glutamate transporter expression in both developing axons and glia, which is consistent with a tight maintenance of extracellular glutamate in the zones where axons and glia meet. Conjunctions between developing axons and their ensheathing oligodendrocytes and surrounding astrocytes may therefore be analogous to neuronal synapses, where release and clearance of extracellular glutamate is essential for cell–cell interaction. Two recent studies have reported vesicular-type glutamate release from axons onto neighboring glial cells (Kukley et al., 2007; Ziskin et al., 2007). The current study confirms for the first time that functional glutamate reuptake mechanism exists within a central white matter tract, adding further to the weight of evidence for synaptic-type glutamatergic signaling between axons and glia.

ACKNOWLEDGMENTS

The authors thank the laboratory of Professor David Attwell for the kind gift of GLAST and GLT-1 KO animals.

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