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Localisation of novel forms of glutamate transporters and the cystine-glutamate antiporter in the choroid plexus: Implications for CSF glutamate homeostasis

Aven Lee[a], **Ashley R. Anderson**[a], **Andrew J. Rayfield**[a], **Melissa G. Stevens**[a], **Philip Poronnik**[b], **James S. Meabon**[c], **David G. Cook**[d,e], and **David V. Pow**[a,*]

[a]The University of Queensland Centre for Clinical Research, Qld 4029, Australia

[b]School of Medical Sciences, RMIT University, Victoria, Australia

[c]Department of Psychiatry, University of Washington School of Medicine, Seattle, WA, United States

[d]VA Medical Center Geriatric Research Education and Clinical Center, Department of Medicine, School of Medicine University of Washington, Seattle, WA, United States

[e]VA Medical Center Geriatric Research Education and Clinical Center, Department of Pharmacology, School of Medicine University of Washington, Seattle, WA, United States

Abstract

The choroid plexus is a structure within each ventricle of the brain that is composed of fenestrated vessels surrounded by secretory epithelial cells. The epithelial cells are linked by tight junctions to create a permeability barrier. The epithelial cells are derived from neuroectoderm, and are thus defined by some authors as a subtype of macroglia. Glutamate is a tightly regulated substance in the CSF, as it is in the rest of the brain. In the brain macroglia express multiple sodium dependent and independent glutamate transporters and are the main regulators of extracellular glutamate. However, the identities of the transporters in the choroid plexus and their localisations have remained poorly defined. In this study we examined the expression and distribution of multiple splice variants of classical sodium-dependent glutamate transporters, as well as the cystine-glutamate antiporter, and the PDZ protein NHERF1, (which acts as a molecular anchor for proteins such as the glutamate transporter GLAST). We identified three forms of sodium-dependent transporters (GLAST1a, GLAST1c and GLT1b) that are expressed at the apical surface of the epithelial cells, a location that matches the distribution of NHERF1 and the cystine-glutamate antiporter. We propose that this coincident localisation of GLAST1a/GLAST1c/GLT1b and the cystine-glutamate antiporter would permit the cyclical trafficking of glutamate and thus optimise the accumulation of cystine for the formation of glutathione in the choroid plexus.

Keywords

EAAT; GLAST; GLT1; Glutamate; Transporter; CSF

[*]Corresponding author at: The University of Queensland Centre for Clinical Research, The University of Queensland, Building 71/918, RBWH, Brisbane, Qld 4029, Australia. Tel.: +61 7 33466012. d.pow@uq.edu.au (D.V. Pow).

1. Introduction

The choroid plexus is typically described as a secretory structure that is present in each of the ventricles of the brain, and which is derived from the neuroectoderm, with fenestrated blood vessels being ensheathed by a layer of epithelial cells. These epithelial cells are polarized, the apical surface being characterised by the possession of microvilli and the polarized expression of a variety of transporters such as the Na/HCO_3 co-transporter (Schmitt et al., 2000) and aquaporin 1 and possibly aquaporin 4 (Speake et al., 2003). These transporters and channels are the main mediators of the secretion of cerebrospinal fluid (CSF).

Differential composition of CSF is both a consequence of the secretory activity of the choroid plexus and the possession of functional segregating barriers, including the blood–brain barrier and the barrier at the boundary of the circumventricular organs. Each of these barriers ensures the anatomical and biochemical segregation of brain, blood and CSF compartments.

One molecular species that is very highly regulated in the brain is glutamate. Whilst glutamate is abundant in blood (around 150 μM) the concentration in CSF is around 10 μM (Vannucci et al., 1999) and in the brain extracellular milieu it is around 2 μM (Lerma et al., 1986), reflecting both the presence of permeability barriers and effective transporters which together ensure very efficient regulation.

Glutamate is an abundant neurotransmitter, and its effective clearance is determined largely by the actions of sodium-dependent glutamate transporters expressed by glial cells. Another role for glutamate is in the biosynthesis of other substances, especially the glutamate-containing tri-peptide glutathione (GSH), which is abundant in the choroid plexus (Anderson et al., 1989). GSH is formed from glutamate, cysteine (derived from cystine) and glycine. Cystine is typically accumulated into cells using the cystine-glutamate antiporter (CGAP; McBean and Flynn, 2001; Pow, 2001). This protein consists of two parts, xCT (the specific part of the CGAP) and 4F2 heavy chain (4F2hc) (Sato et al., 1999). The accumulation of cystine requires the concomitant export of glutamate to provide the motive force that drives cystine uptake. Since released glutamate is potentially excitotoxic and is needed as a substrate for GSH synthesis itself, it must be re-uptaken, presumably via a classical sodium-dependent glutamate transporter. This raises the possibility that co-expression of classical sodium-dependent glutamate transporters and the CGAP might be required to achieve the homeostasis and compartmentation of glutamate, cystine and GSH. One alternative mechanism for the accumulation of cysteine is via the sodium-dependent glutamate transporters, particularly Excitatory Amino Acid Carrier 1 (EAAC1), also known as Excitatory Amino Acid Transporter 3 (EAAT3), which may transport cysteine, rather than cystine (Hayes et al., 2005).

Surprisingly there is a relatively limited literature on glutamate transport in the choroid plexus. Functional uptake assays indicate a generalized tendency for the removal of glutamate from the CSF (Kim et al., 1996; Nixon, 2008). These data indicate the presence of transporters on the apical surface of the choroid plexus. There is also evidence for a low-level expression of transporters on the basolateral (blood-facing) surface, as uptake studies in the sheep show there can be a very limited accumulation of glutamate from the blood into the CSF, probably via a sodium-independent mechanism (Segal et al., 1990). The identification of transporters mediating these measured fluxes and the polarity of their expression relative to the CSF and blood is poorly described. Three scenarios might be envisaged, the first being that glutamate is transported from CSF to blood, through the epithelial cells, an arrangement that would require transporters located on the apical surfaces

of the epithelial cells (that are apposed to the CSF) and basal transporters that are apposed to the blood vessels. This scenario would require the asymmetric functioning of the transporters in the epithelial cells with apical transporters directing inward transport and basolateral types directing outward transport. The simplest conceptual arrangement that could achieve this would be via expression of sodium-dependent transporters on the apical surface of the epithelia and the CGAP on the basolateral surfaces. Whilst this first scenario is a mechanistically appealing arrangement, other plausible arrangements exist. Sato et al. (2002) have previously suggested that the CGAP might function to maintain the redox state of the CSF, by regulating the cystine/cysteine ratio in the CSF. Transport of cystine out (or into) the CSF would require that the CGAP was localised to the apical surface and that the sodium-dependent glutamate transporters are localised either to the basolateral surface or to the apical surface. A localisation of the sodium-dependent glutamate transporters to the basal surface would potentially yield an influx of glutamate from the blood into the epithelial cells, which does not accord with the general view of the choroid removing glutamate from the CSF. Conversely, if the sodium-dependent types were expressed on the apical surface, this would allow the removal of glutamate from the CSF, including the glutamate released from the epithelia as a consequence of cystine uptake by the CGAP.

Early studies examining mRNA distributions by in situ hybridisation and protein expression by immunocytochemistry typically failed to localise or identify the choroid plexus glutamate transporters, and led to the broad conclusions that the dominant glial cell glutamate transporter types including Glutamate Aspartate Transporter 1 (GLAST, also called EAAT1) and Glutamate Transporter 1 (GLT1, also called EAAT2) were absent from the choroid plexus (Schmitt et al., 1996; Berger and Hediger, 2000). Later studies by Berger et al. (2005) and Holmseth et al. (2009) also failed to detect transporter proteins such as GLT1b in the choroid plexus. The reasons for the lack of success in localising glutamate transporters in a tissue that can transport glutamate, is puzzling. In some instances they may simply have been overlooked; thus Furuta et al. (1997) present images in the developing rat brain where GLT1 and EAAC1 immunoreactivity may be present in the choroid plexus (their Fig. 3A, B) but this is not commented upon in their text. In most systematic surveys of the brain (for example, Rothstein et al., 1994) the choroid plexus is not mentioned, so the presence or absence of glutamate transporters in the choroid plexus cannot be determined in such studies. Recently Beschorner et al. (2009) have indicated that expression of immunocytochemically detectable EAAT1 was not normally evident in the normal choroid plexus, and that indeed the presence of such was indicative of choroid plexus tumours. Conversely one paper has suggested that EAAC1 might be expressed in the choroid plexus (Kugler and Schmitt, 1999), which accords with the images presented by Furuta et al. (1997) and accords also with the evidence for mRNA for EAAC1 in the ependymal cells (Schmitt et al., 1997). There is good evidence for the high expression level of the cystine-glutamate antiporter in the choroid plexus, at the mRNA level (Sato et al., 2002) but the immunocytochemical evidence to date has been ambiguous with a localisation of an unexpectedly small 40 kDa species to what appears to be a nuclear compartment rather than cell plasma membranes (Burdo et al., 2006). With the recent findings of multiple alternate splicings of glutamate transporters such as GLT1 and GLAST in the brain and other tissues (see Lee and Pow, 2010) for summary and nomenclatures) we have sought to evaluate the expression patterns of glutamate transporters, particularly their splice variants, in the choroid plexus, at the mRNA and protein levels and to determine the polarity of such expressions in the epithelial cells. At least one glutamate transporter (GLAST) is anchored via the interactions of a PDZ-motif, using the PDZ scaffold protein Sodium-Hydrogen Exchange Regulatory Factor 1 (NHERF1) (Lee et al., 2007). Accordingly, given the need for the polarity of glutamate transporter expression in the choroid plexus, the expression of NHERF1 and NHERF2 were examined. Similarly, the distribution of the cystine-glutamate antiporter was examined by immunocytochemistry as well as by Western blotting and PCR.

2. Methods

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, the NHMRC code and with ethical permission from the University of Queensland Animal Ethics Committee. Dark Agouti rats were maintained in our own colony. GLT1 knockout mice and matched control mice were also analysed to confirm specificity of GLT1 antibodies. GLAST and CGAP knockout mice exist (Sato et al., 2005) but their availability is restricted and we were unable to access such animals as additional immunocytochemical controls in this study.

2.1. Isolation of choroid plexus

For studies that required isolation of the choroid plexus, including Western blotting, PCR and uptake studies, the choroid plexus was obtained from the lateral ventricles of rats. Briefly, adult (200–250 g) male and female Dark Agouti rats (n = 23) were deeply anaesthetised with sodium pentobarbital (Lethobarb; 150 mg kg I.P.) and the brains rapidly removed. Brains were bisected in the sagittal plane and each hippocampus reflected back to reveal the lateral ventricle. The choroid plexus was then removed from each lateral ventricle by grasping such with fine watchmakers forceps. This simple and rapid protocol ensured there was no contamination of the choroidal tissues with other brain components. Fig. 1 depicts examples of the choroid plexus extracted via this method.

2.2. Transport studies

To evaluate which cellular compartments expressed functional glutamate transporters including the cystine-glutamate antiporter, uptake of two different non-metabolisable glutamate analogues, D-aspartate and DL-alpha aminoadipic acid (AAA) was examined. D-Aspartate is widely presumed to be a ligand for all functional sodium-dependent glutamate transporters (but not the cystine-glutamate antiporter). Detection of D-aspartate uptake was carried out according to our standard methods (Barnett and Pow, 2000; Pow and Barnett, 1999; Williams et al., 2006). Briefly entire individual choroid plexuses were placed into oxygenated physiological media (Ames media, Sigma) or Ames media containing 20 μM D-aspartate, at 35 °C for 30 min. In the isolated choroid plexus the major surface that is exposed to the D-aspartate is the CSF-facing face of the epithelia; the basal surface would only be accessible to molecules that diffuse along the internal vessels and spaces of the choroid plexus. Tissues were then removed, washed for 1 min in oxygenated Ames media at 35 °C to remove any free D-aspartate, and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 1 h. Tissues were subsequently dehydrated, embedded in Araldite resin and immunocytochemistry performed for D-aspartate using a specific antibody to D-aspartate, as previously described. Control sections that had not been exposed to D-aspartate (exposed to normal Ames media alone prior to fixation or fixed immediately after removal from the animal) were also evaluated to determine if any endogenous D-aspartate could be detected. Methodological controls included the use of dihydrokainic acid (DHK), which is a selective GLT1 uptake inhibitor and TBOA, which is a non-selective inhibitor of uptake by sodium-dependent glutamate transporters.

Aminoadipic acid (AAA) is thought to be a selective substrate for the CGAP (Pow, 2001). 20 μM AAA was applied to individual isolated choroid plexuses using the same methods as for D-aspartate uptake, uptake being revealed using an antibody to AAA (Pow, 2001).

2.3. RT-PCR screening of choroid plexus for EAATs, NHERF1 and CGAP

Total RNA was isolated from choroid plexus, whole brain, and retina of adult Dark Agouti rats (n = 13) using TriZol® reagent (Invitrogen, Mulgrave, Victoria, Australia). Total RNA (5 μg) of each sample was reverse-transcribed into complementary DNA using SuperScript

III (Invitrogen), followed by digestion with Ribonuclease H (Invitrogen), according to the manufacturer's instructions. An aliquot of the RT reaction mixture (1 μl) was then used in PCR (final volume 50 μl) consisting of 2 mM dNTP, 0.2 mM sense and antisense primers, 1.5 mM $MgCl_2$, and 2.5 U BIOTAQ DNA polymerase (Bioline Pty Ltd, Alexandria NSW, Australia) in 1× PCR buffer. PCRs were performed using the following conditions: initial denaturation at 95 °C for 2 min followed by 35 cycles of amplification (95 °C for 30 s, 60–62 °C for 30 s and 72 °C for 1 min). The reaction products were separated on 1.5% agarose gels and visualised by staining with 0.5 μg/ml ethidium bromide (Sigma–Aldrich, Castle Hill, Australia). Sequences of sense and antisense primers are provided in Table 1.

In addition to PCR analysis, additional characterisation of GLAST in the choroid plexus was performed by cloning and sequencing using standard methods. The PCR products were excised from the gel, purified using a gel extraction kit (Qiagen Pty Ltd, Doncaster VIC, Australia) and subcloned into the pcDNA3.3-Topo vector (Invitrogen) according to the manufacturer's instructions and sequenced in both directions by the Australian Genome Research Facility (Brisbane, Qld, Australia).

2.4. Western blotting

Rat choroid plexus tissue plus rat brain and rat retina from 27 adult Dark Agouti rats were each homogenized in lysis buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche Diagnostics, Castle Hill, NSW, Australia). After gentle rotation for 3 h at 4 °C, homogenates were centrifuged at 17,000 × *g* for 1 h at 4 °C and the supernatant collected. Protein lysate (~10 μg) was dissolved in SDS sample buffer, separated on a 7% SDS–polyacrylamide gel and then transferred to nitrocellulose membrane (Pall, Cheltenham, Victoria, Australia) by electroblotting. Blots were incubated in blocking buffer (5% non-fat milk, 20 mM Tris (pH 7.5), 150 mM NaCl and 0.1% Tween-20) for 2 h and then incubated overnight at 4 °C in fresh blocking buffer containing primary antibodies against the EAATs or CGAP. Antibodies are listed in Table 2. Blots were then washed four times with Tris–NaCl–Tween buffer, then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and washed again. Immunoreactive proteins were detected by enhanced chemiluminescence using the SuperSignal® West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL, USA). Preabsorption of antisera (50 μg of antigen peptide per milliliter of diluted antiserum) was used to confirm the specificity of each antiserum.

2.5. Primary antibodies against transporters

The primary antibodies to glutamate transporters used in this study were all raised in this laboratory and have been extensively characterised in prior publications (Macnab and Pow, 2007a,b; Macnab et al., 2006; Rauen et al., 2004; Reye et al., 2002). The CGAP antibody was raised against a unique peptide sequence corresponding to residues 475–499 of the rat cystine-glutamate antiporter (NCBI Reference Sequence: NP_001101143.2.1). This is referred to as $CGAP_{475}$. A second antibody against the peptide sequence corresponding to amino acid residues 12–36 of the mouse CGAP (NCBI Reference Sequence: NP_036120.1) (referred to as $CGAP_{12}$) was used to confirm the results obtained with the $CGAP_{475}$ antibody.

In dot blots both CGAP antisera detected their appropriate target sequences and in Western blotting they each detected a protein of ~55 kDa, which is the predicted molecular weight of this protein.

Methodological controls included the use of pre-immune sera and sera that had been absorbed with the target peptide (for details see Lee et al., 2010).

Additional antibodies against the GABA transporters GAT1 and GAT4 (Pow et al., 2005) were also used as methodological controls, as only GAT2 is thought to be expressed by the choroid plexus (Conti et al., 1999).

The NHERF1 and NHERF2 antibodies were kindly provided by Dr C. Yun. For details of their use see Paquet et al. (2006) and Lee et al. (2007).

2.6. Immunocytochemistry

Immunoperoxidase labelling was performed as previously described using standard methods (Pow et al., 2004). Briefly, brains from adult Dark Agouti rats (n = 8) were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, then dehydrated through a graded series of water/ethanol solutions, cleared in xylene and embedded in paraffin wax. Serial sections (8 μm in thickness) were cut on a Leica rotary microtome and mounted onto silanated microscope slides. Sections were dewaxed with xylene and rehydrated through a graded series of ethanol/water solutions and antigen recovery was routinely performed using Revealit-Ag antigen recovery solution (ImmunoSolution, Newcastle, NSW, Australia). Sections were pre-treated with 3% hydrogen peroxide in methanol for 10 min (during the rehydration process) to inhibit any endogenous peroxidase activity. All sections were blocked in 0.5% bovine serum albumin/0.05% saponin/0.05% sodium azide in 0.1 M sodium phosphate buffer for 30 min before primary antibodies were applied. Biotinylated secondary antibodies and streptavidin–biotin–horseradish peroxidase conjugates were subsequently applied at a dilution of 1:300. Labelling of sections was revealed using 3,3′-diaminobenzidine as a chromogen, and sections were mounted using DePex. Pre-absorption of antisera (50 μg of peptide per 1 ml of diluted antiserum) was always used to confirm the specificity of each antiserum (data not shown). To validate the specificity of GLT1 primary antibodies, GLT1 knockout mice were also examined. Such mice were immunolabelled by immunofluorescence methods, using the primary antibodies at comparable dilutions. Briefly, frozen sagittal brain sections obtained from GLT-1 WT and GLT-1 KO (p18–21) were air dried (15 min) and acetone fixed (15 min).

Tissue fixation was followed by an additional air dry (10 min) and tissue rehydration via PBS submersion washes (3 × 3 min). Slides were then blocked for 1 h in 10% milk/PBS and incubated in primary antibodies overnight at 4 °C. The next day, slides were PBS rinsed (4 × 3 min) and incubated with goat anti rabbit Alexa-488 (3 h; 2 μg/ml in 1% BSA/PBS; Molecular Probes, #A11070, Oregon, USA). Slides were then washed and finally mounted in 1:1 glycerol:H_2O.

2.7. Brightfield microscopy

Imaging was performed using a Nikon 80i microscope equipped with a Olympus DP70 camera. Images were imported into Adobe Photoshop for minor contrast and colour balance adjustments prior to composition of plates using Adobe Freehand.

2.8. Confocal fluorescence microscopy

Images were obtained using a Leica confocal microscope (Leica microsystems, Bannockburn, IL) and formatted using Photoshop (Adobe Systems, San Jose, CA, USA). For each condition (GLT-1 WT versus GLT-1 KO) microscopic images were acquired and digitally processed under identical conditions. Digital image processing was limited to linear brightness and contrast adjustments that were performed identically on experimental and control images. For presentation the images were converted to grayscale.

3. Results

In this study the choroid plexus was readily isolated from the lateral ventricles using the method indicated, the tissues being isolated with good structural integrity and without adherent brain tissues or other contaminating material (Fig. 1A, B).

3.1. Detection of glutamate transporters using PCR

PCR using the primer sets indicated (Table 1), demonstrated that we are able to detect mRNA for EAATs 1–5 in brain or retina (Fig. 2A). By comparison, whilst EAAT1 (GLAST) mRNA was detectable in the choroid plexus, only an extremely faint signal was obtained for GLT1a (the originally described form of GLT1, which is also known as EAAT2). No signals were obtained for EAAT3 (EAAC1), EAAT4 or EAAT5 (Fig. 2A). Because GLAST exists as multiple splice variants (GLAST1a, GLAST1b and GLAST1c; see Fig. 3 for details), PCR was performed using specific primer sets for each of the known forms of GLAST. We demonstrated (Fig. 2B) that message for GLAST1a and GLAST1c is present but message for GLAST1b is absent. This was not a methodological failure as GLAST1b message was detectable in brain (Fig. 2B). The identity of the forms of GLAST detected by PCR was further verified by sequencing, and confirmed that these forms of GLAST were indeed present. It was notable that they included the expected sequences that would form the N-terminal region of the translated proteins, a point that was significant due to later issues with detecting this part of the GLAST proteins by immunocytochemistry or Western blotting.

Similarly, because GLT1 is present as multiple carboxyl terminal splice variants in addition to GLT1a, we performed PCR that selectively identifies the coding sequences for the carboxyl terminal regions of GLT1b and GLT1c. We demonstrate (Fig. 2C) the presence of mRNA for GLT1b but not GLT1c. Internal exon skipping splice variants of GLT1 also exist (Lee and Pow, 2010) and so we performed PCR for these known variants. We identified the presence of exon-9 skipping GLT1 and subsequently demonstrated, using specific primer sets that this product corresponds to an exon-9-skipping form of GLT1b (Fig. 2D). Similarly, we have examined the expression of the cystine glutamate antiporter. We demonstrate the presence of a strong signal for this antiporter in both brain and choroid plexus (Fig. 4A). Expression of the scaffold proteins NHERFs 1 and 2 were also examined by PCR (since GLAST is thought to be anchored via NHERF1). We demonstrate (Fig. 4B) strong signals for both NHERFs in brain and choroid plexus.

3.2. Detection of glutamate transporter proteins by Western blotting

To confirm that mRNA species were being translated into protein, Western blotting was performed. We demonstrate (Fig. 5A) that whilst strong signals were obtained for the amino terminal region of GLAST in brain tissues, no signals were obtained from choroid plexus. Conversely, Western blots for GLAST1a and GLAST1c revealed strong signals in choroid plexus (Fig. 5B), whilst GLAST1b, which was present in brain, was not detectable in the choroid plexus. Western blotting for GLT1 revealed no detectable signals for GLT1a (data not shown) but strong signals for GLT1b (Fig. 5C). Similarly, Western blots were performed for exon-9 skipping GLT1 (Fig. 5D). A strongly labelled band was present, indicating that an exon-9 skipping form of GLT1 was present. Western blots were performed for each of the other EAATs (EAAT3, EAAT4 and EAAT5) but no signals were detected (data not shown). Conversely, Western blotting for the cystine glutamate antiporter (Fig. 5E), revealed labelling of a single band at ~55 kDa, a size corresponding to the predicted mass of the cystine-glutamate antiporter.

3.3. Immunocytochemical localisation of transporter proteins

To determine at the anatomical level the localisations of the protein species detected by Western blotting, immunocytochemistry was performed. Examples are given for choroid plexus from the lateral ventricles and the fourth ventricle, however for each marker no fundamental differences in labelling were observed for choroid plexus from each ventricle. We demonstrate that whilst N-terminal GLAST immunoreactivity was absent from the choroid plexus (Fig. 6A), GLAST1a (Fig. 6B, C) and GLAST1c immunoreactivities (Fig. 6D) were present at the apical surfaces of the epithelial cells. Similarly whilst labelling for GLT1a was absent from the choroid plexus (Fig. 7A), labelling for GLT1b (Fig. 7B, C) and for exon 9 skipping GLT1 (Fig. 7D) was demonstrated at the apical surface of the epithelia. To confirm that GLT1b immunolabelling was attributable to GLT1 rather than another unknown protein immunolabelling was also performed on GLT1 knockout ice and wild type control mice. Strong GLT1b immunoreactivity was evident in the choroid plexus of control mice (Fig. 8A) but was absent from the choroid plexus of knockout mice (Fig. 8B). Immunolabelling for the CGAP using $CGAP_{475}$ revealed strong signals associated with the apical surface of the epithelial cells (Fig. 9A) and this was replicated with the $CGAP_{12}$ antibody (Fig. 9B). By comparison, controls such as the use of pre-immune sera (Fig. 9C) resulted in an absence of any specific labelling. Similarly, labelling for other EAATs such as EAAC1 (EAAT3) was not evident in the choroid plexus, though labelling for EAAC1 was observed in the adjacent ependymal cells as well as neuronal elements (Fig. 10A). As part of our controls we examined GABA transporter immunolabelling. We demonstrate that the GABA transporters GAT4 and GAT1, which should not be expressed by the choroid plexus, were absent (Fig. 10B), thus confirming that our immunocytochemical methods were not generally subject to artifacts that could yield spurious non-specific labelling of the apical surface of the epithelia.

Because GLAST is typically anchored to NHERF1 (but not NHERF2), immunolabelling was performed for NHERFs 1 and 2. Immunolabelling for NHERF1 (Fig. 11A, B) was very strong at the apical surface of the choroid plexus epithelia, whereas NHERF2 labelling was restricted to the capillary endothelial cells (Fig. 11C).

3.4. Transport studies

To verify that functional transporters were present in the epithelia, AAA and D-aspartate uptake experiments were performed. AAA is a substrate for the CGAP and was accumulated into the epithelial cells (Fig. 12A). D-Aspartate is a substrate for all EAATs. Similarly D-aspartate was accumulated into the epithelial cells (Fig. 12B). Uptake of D-aspartate was markedly reduced by TBOA (Fig. 12C) and to a lesser extend by DHK (Fig. 12D), suggesting that a significant proportion of choroidal glutamate transport was mediated via a non-GLT1 (EAAT2) type transporter.

4. Discussion

In this study we find that two sodium dependent glutamate transporters and the CGAP are expressed by the choroid plexus epithelia. Our finding that the CGAP is localised solely to the apical surface of the choroid plexus supports the view, as suggested by Sato et al. (2002), that the CGAP is present to regulate CSF cystine and/or be used for providing cystine for the synthesis of glutathione in choroidal epithelial cells, rather than to import cystine from the blood. Confirmation of this result by a second antibody, and the observed uptake of AAA, which is predominantly exposed to the apical surface of the epithelia in our isolated choroid plexus preparations, strongly supports our conclusion that the CGAP is localised to the apical rather than the basolateral surface of the choroidal epithelia.

4.1. Is EAAC1 a choroidal glutamate transporter?

Our finding, that EAAC1 is not detectable at the mRNA level by PCR and not detectable at the protein level by immunocytochemical techniques is in contrast to the earlier findings by Kugler and Schmitt (1999) who observed immunolabelling for EAAC1 in the cytoplasm of choroidal epithelial cells. Interestingly, Furuta et al. (1997) show, but do not comment upon what appears to be EAAC1 labelling in the choroid plexus in E18 rats. Accordingly, the differing results may simply reflect developmental changes in the choroid plexus, and this awaits further investigation. We conclude that if EAAC1 is present in the adult rat choroid plexus, it is not present at significant levels, and thus is unlikely to contribute either to glutamate uptake or to cysteine uptake.

4.2. Sodium-dependent glutamate transporters expressed by the choroid plexus

In this study we have identified two types of sodium dependent glutamate transporter proteins in the choroid plexus epithelial cells, which are variants of GLAST and GLT1. Both of these proteins are unusual in that they are present as exon-skipping types. Thus GLAST is present as exon 3 skipping and exon 5/6 skipping forms, referred to as GLAST1a and GLAST1c, respectively. Intriguingly, the N-terminal region of GLAST appears to be missing, as immunolabelling with N-terminal-directed antibodies yields a negative result, which could be interpreted as indicating the epitope is either modified or has been cleaved off.

4.3. Does functional GLAST lack N′ and C′ epitopes?

We have cloned and sequenced the forms of GLAST that are expressed by the choroid plexus, and note that they contain intact N-terminal regions, indicating that absence of immunoreactivity for the N-terminal region is evidence for post translational modification, possibly by the cleavage of the N-terminal region. Similar phenomena been observed elsewhere in the brain (Sullivan et al., 2007) and in non-nervous tissues such as testis (Lee et al., 2010), and these accord with key earlier studies which show that immunoreactivity for N- and C-terminal regions of GLAST diminishes when GLAST transport activity is up-regulated by pharmacological manipulation of PKC (Susarla et al., 2004). Other studies have demonstrated that de-glycosylated GLAST (detected with N-terminal antibodies) often runs at a much smaller molecular weight than would be predicted; thus Schmitt et al. (1997) in their Fig. 4, show an approximately 10 kDa disparity between the expected and the observed size of GLAST, with further differences between hippocampus and cerebellum, possibly indicating the cleavage of GLAST. Conversely, the lack of any GLAST protein at the predicted size might suggest that all the protein is alternately spliced, which seems unlikely. We suggest that the presence of forms of GLAST that do not exhibit N-terminal immunoreactivity is indicative of the presence of active transporting forms of these transporters which have had their N-terminal regions cleaved or modified in a manner which renders them undetectable to N-terminal directed GLAST antibodies. This appears to be supported by the D-aspartate transport studies, which demonstrate the uptake of D-aspartate into the choroidal epithelial cells. The inhibition of uptake by TBOA (which blocks all EAATs) and to a lesser extent DHK (which inhibits GLT1), is indicative of uptake of D-aspartate (and thus by inference glutamate), by both a GLT-like transporter, and to a greater extent by a non-GLT1-type transporter (which we assume to be GLAST1c and GLAST1a).

4.4. GLT1 in the choroid plexus

In this study we demonstrate that an extremely faint band is detected by PCR for GLT1a, but protein could not be detected, suggesting the level of expression was below detection thresholds for antibody methods. By contrast we were readily able to detect immunoreactivity corresponding to an exon-9 skipping form of GLT1, and

immunoreactivity for GLT1b. Similarly we were able to detect an exon-9-skipping form of GLT1b by PCR. The presence of GLT1b immunoreactivity has previously been noted in the epithelial cells, albeit without clarification as to where in the epithelia it was localised (Schmitt et al., 2002) though this was not confirmed by Holmseth et al. (2009), who reported a lack of GLT1a or GLT1b immunoreactivity in the choroid plexus. Curiously, Berger et al. (2005) reported signals for GLT1b in the choroid plexus using in situ hybridisation and relatively high probe concentrations, but referred to these as non-specific, despite the much lower signals observed using sense probes. An alternative view is that there are modest levels of GLT1b message in the choroid plexus, resulting in modest in situ hybridisation signals. In this study we present evidence in support of the expression of GLT1b in the choroid plexus, at both the mRNA and protein level. The lack of immunolabelling that we describe in GLT1 knockout mice supports the view that our antibody specifically detects GLT1b, an important point as there have been other GLT1b antibodies which are relatively non-specific (Chen et al., 2002; cited in Chen et al., 2004). This study expands and refines the observations of Schmitt et al. (2002) both by defining the localisation of GLT1b protein to the apical surface of the epithelia and by our finding that the form of GLT1 that is expressed appears to be a novel exon-9 skipping form.

4.5. Technical issues associated with detecting glutamate transporters

We note that the glutamate transporter literature is still in some disarray as to the precise localisations or even expression of known types of glutamate transporters in the brain. Early studies such as those of Berger and Hediger (1998) suggested extensive neuronal expression of transporters such as GLT1, in areas such as cortex, which appears at odds with later studies which report a predominant glial expression. A hint that they may not be incorrect is evident in Furness et al. (2008) where an unusually high level of GLT1-mediated glutamate transport is evident in synaptosomes. One plausible explanation, raised by Berger and Hediger (1998), but seemingly ignored in the subsequent literature, is that some commonly used antibodies do not detect some forms of GLT1, an explanation that would accord with our findings where we detect GLT1b protein and mRNA in the choroid plexus, whilst at least some other groups do not do so. A similar logic applies also to GLAST, where, as we have noted, there is potential for N and possibly C-terminal cleavage of the protein, or modifications of these terminal regions, yielding proteins that are not detectable by at least some of the commonly used antibodies in prior studies. Other technical factors that might influence capacity to detect glutamate transporters in brain tissues have been described in the literature such as the anaesthetics and different blocking agents (Holmseth et al., 2009). Similarly, the need to very effectively permeabilise thick sections, which have often been used in earlier studies (rather than our lightly fixed thinner sections which have already been permeabilised during the wax embedding process) in order to access the intracellular epitopes studied, may also be key factors in differential outcomes of our studies relative to those of others.

This study has demonstrated the expression of both protein using selective antibodies and mRNA expression by PCR using specific primers for each of the forms of GLT1 or GLAST that we describe. Accordingly we suggest that exon-skipping forms of GLAST and GLT1b are present in the choroid plexus, where they are expressed at the apical surface of the epithelia. By contrast we were unable to detect any glutamate-transporting proteins at the basolateral surface, a finding that suggests most glutamate flux is likely to occur between the CSF and the epithelia rather than the blood and the epithelia.

4.6. Anchoring glutamate transporters to the apical domains of choroidal epithelial cells

It is known that GLAST is anchored at the surface of glia such as astrocytes via the scaffold protein NHERF1. Our studies confirm that NHERF1 is abundant in the choroid plexus, and

is localised to the apical face, a location that would be commensurate with it anchoring GLAST. By contrast NHERF2 was restricted to the capillary endothelial cells, suggesting that it is not implicated in glutamate transport (since these capillaries are fenestrated), but may play a role in anchoring other proteins in the endothelial cells.

Our internal controls (including the use of preimmune and preabsorbed sera) reinforce the view that we are not artifactually detecting transporters at the apical surface of the epithelia due to methodological anomalies that give rise to spurious staining at this surface. Similarly our use of antibodies to GAT1 and GAT4, which did not yield labelling in the choroid plexus, reinforce the view that the choroid plexus is not simply expressing transporter proteins in a promiscuous fashion. Accordingly the anatomical localisations we describe are probably effective indicators of the functional compartmentalisation of the epithelial cells, and thus the probable polarity of transport of glutamate and cystine by the choroid plexus.

The overall conclusion of this study is that two forms of GLAST and a novel form of GLT1 are present in the choroid plexus and may mediate glutamate transport into the choroid plexus. We confirm that the CGAP is expressed, but suggest, that it is localised to the apical rather than to the basolateral face of the epithelial cells. This finding is of interest because it is known that rBAT, which can substitute for 4F2hc as the co-associated component of the functional transporter, is also localised to the apical face of epithelial cells (Wang et al., 2003). Whether the targeting of the CGAP can be changed under differing physiological conditions remains to be determined. We note that a sodium-independent transport of glutamate has been described from blood to CSF (Kim et al., 1996), implying a basolateral localisation of a transporter, possibly the CGAP. However based upon the observed V_{max}, the authors concluded that there was a low capacity for transport into the CSF from the blood, possibly indicating low levels of expression of the CGAP, which might be below our detection thresholds. Similarly, we cannot exclude the possibility that other glutamate transporters may be up-regulated, or conversely down-regulated in response to physiological stressors as we have previously shown a dramatic increase in expression of GLAST1b in response to hypoxia (Sullivan et al., 2007), as well as changes in other glutamate transporters such as GLT1c in response to injurious conditions (Sullivan et al., 2006).

We suggest that the choroid plexus and in particular the transporters we describe, may represent a novel target for pharmacotherapy under conditions where anomalies in CSF glutamate and cystine may be present, such as after strokes.

Acknowledgments

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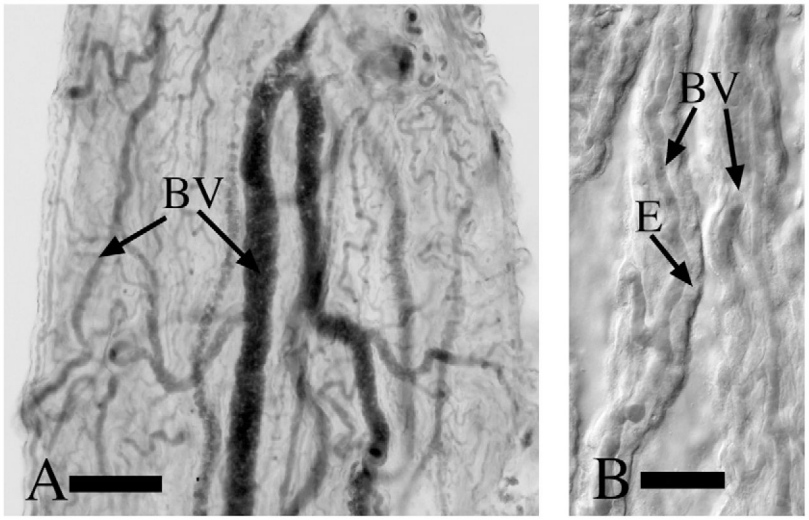

Fig. 1.

Low and high magnification images of examples of isolated choroid plexus used for PCR, Western blotting and uptake experiments. BV indicates the choroidal blood vessels surrounded by epithelial cells (E). Scale bars: (A) 50 μm and (B) 20 μm.

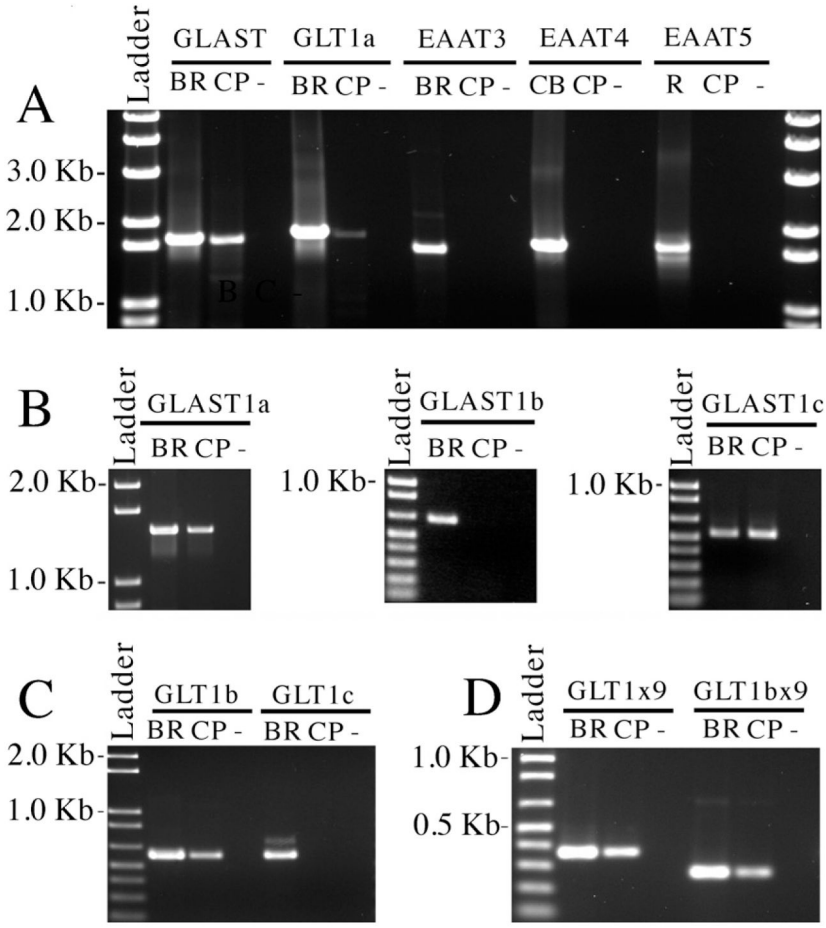

Fig. 2.

Expression of EAATs and EAAT splice variants: cDNAs prepared from rat whole brain (BR), cerebellum (CB), retina (R) and choroid plexus (CP) were used as templates in PCR with specific EAAT primers (as listed in Table 1). A PCR negative control (−) is also shown. An aliquot of each PCR was electrophoresed on 1.5% agarose gel and visualised with ethidium bromide staining. (A) Amplification products corresponding to GLAST and GLT1a but not EAAT3–5 were detected in choroid plexus. (B) PCR products corresponding to GLAST1a and GLAST1c were detected in both brain and choroid plexus, whereas a GLAST1b specific product was only detected in brain. (C) PCR product corresponding to GLT1b was detected in brain and choroid plexus whilst GLT1c specific product was only detected in brain. (D) GLT1 (exon 9-skip) and GLT1b (exon 9-skip) amplification products were detected in both brain and choroid plexus.

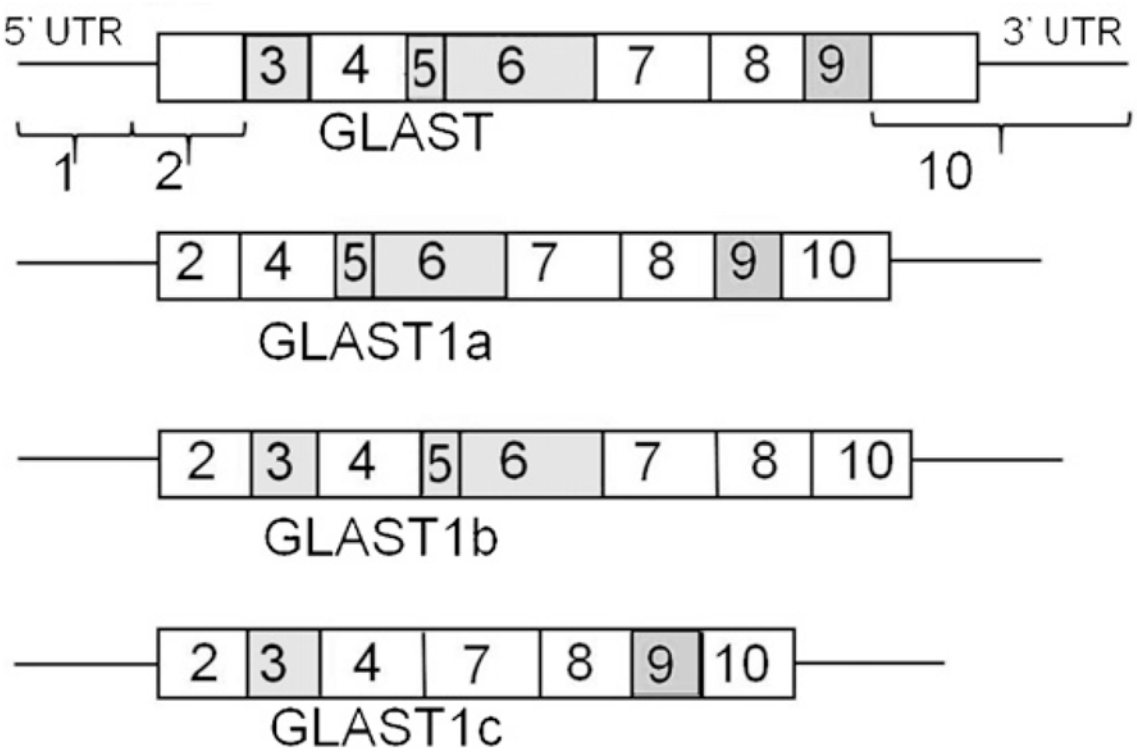

Fig. 3.
Schematic diagram of the exon structures of each of the known forms of GLAST.

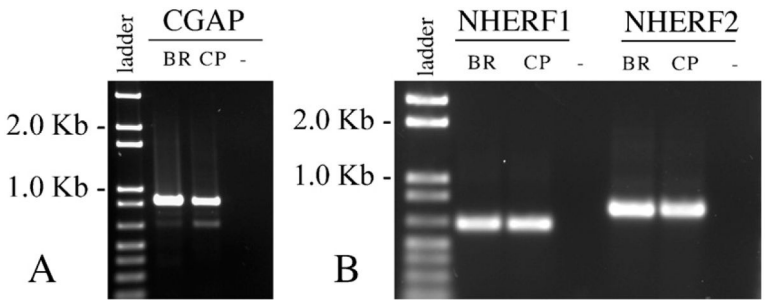

Fig. 4.

(A) Expression of CGAP in choroid plexus. RT-PCR using CGAP specific primers produced amplification product of correct size in both rat brain and choroid plexus. BR, brain; CP, choroid plexus; −, negative control. (B) Expression of NHERF1 and NHERF2 in choroid plexus. RT-PCR using NHERF1 and NHERF2 specific primers produced amplification products of correct size in both brain and choroid plexus. BR, brain; CP, choroid plexus; −, negative control.

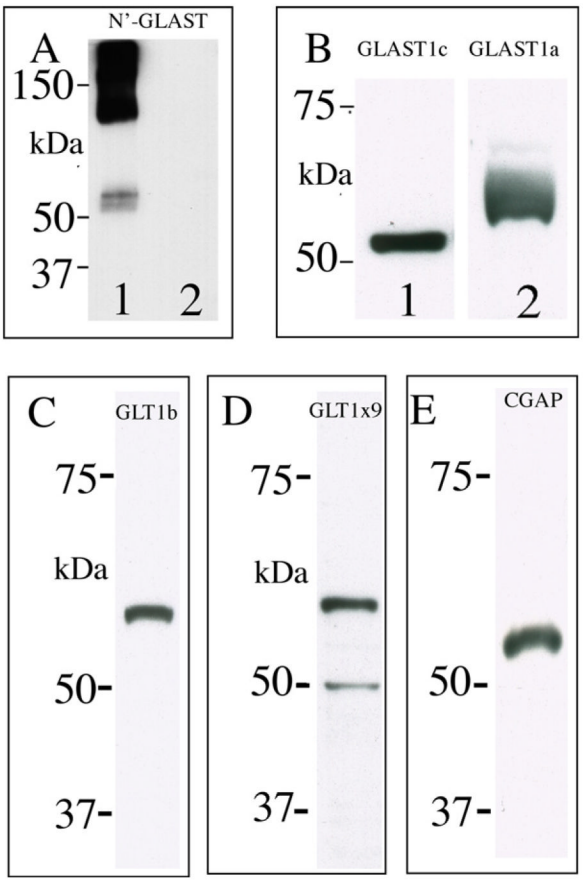

Fig. 5.

(A) Western blotting of brain (lane 1) and choroid plexus (lane 2) using an antibody directed against the N-terminal region of GLAST. Abundant labelling for the monomers and oligomers of GLAST is evident in brain but labelling is absent in the choroid plexus. (B) Western blotting for GLAST1c (lane 1) and GLAST1a (lane 2) demonstrating the presence of both of these forms of GLAST in the choroid plexus. (C) Western blotting for GLT1b. A single strongly labelled band is detected in choroid plexus. (D) Western blotting for exon 9 skipping GLT1. Two bands are detected, the upper band representing labelling for exon 9-skipping GLT1, whilst the lower band represents non-specific labelling due to detection of immunoglobulin heavy chains at ~50 kDa, this being inferred because choroid plexus blots probed only with the secondary antibody also detected this band in some samples, depending on the batch of secondary antibody. (E) Western blotting for the cystine-glutamate antiporter. A single strongly labelled band is evident in blots of choroid plexus.

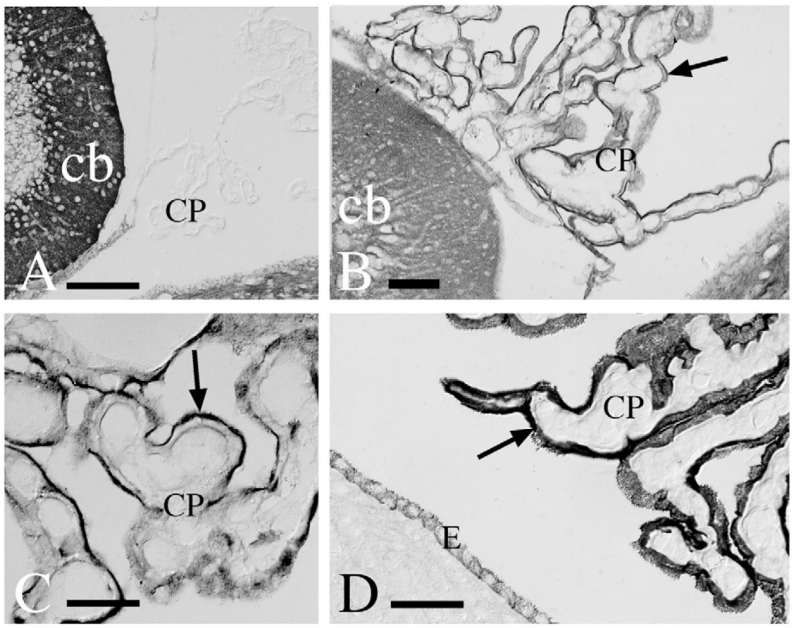

Fig. 6.

Immunolabelling for GLAST in saggital sections that encompass the cerebellum and the choroid plexus in the fourth ventricle, using an N-terminal directed antibody (A), which labels the cerebellum (cb) but not the choroid plexus (CP). Labelling for GLAST1a (B, C) demonstrates labelling of the CP, particularly the apical face of the epithelia (C, arrow). Labelling for GLAST1c (D) is also associated with the epithelial apical surfaces (arrow). Scale bars: (A) 100 μm, (B) 50 μm, (C) and (D) 25 μm.

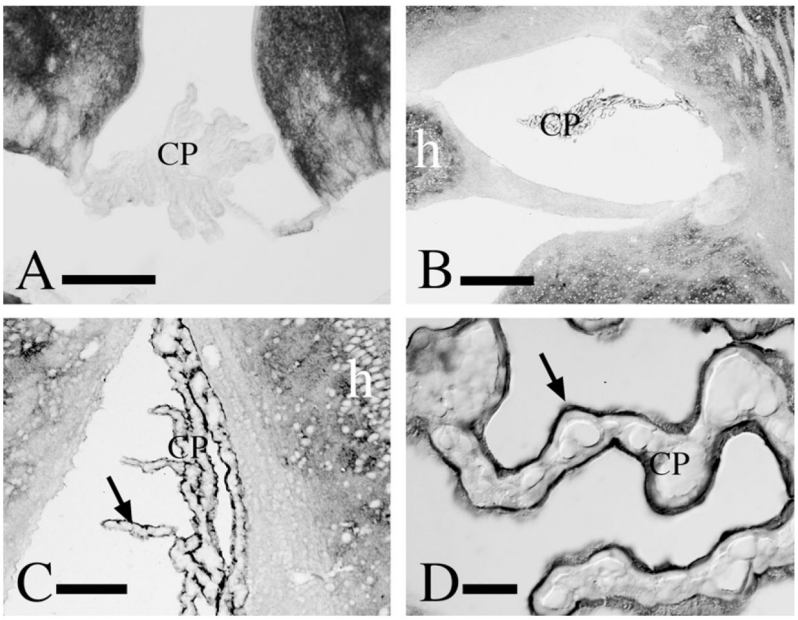

Fig. 7.

Immunolabelling for differing forms of GLT1 in the choroid plexus. (A) Coronal section; GLT1a; labelling is absent from the choroid plexus (CP) at the juncture of the lateral ventricles, though it is abundant in adjacent grey matter. (B, C) Sagittal sections depicting low and high magnification images of labelling for GLT1b; labelling is associated with the apical surfaces of the epithelial cells of the choroid plexus in the lateral ventricles (arrow) as well as in astrocytes in other adjacent grey matter areas such as hippocampus (h). (D) Labelling for exon 9-skipping GLT1; labelling is associated with the apical surface of the epithelial cells (arrow). Scale bars: (A)–(C) 50 μm and (D) 25 μm.

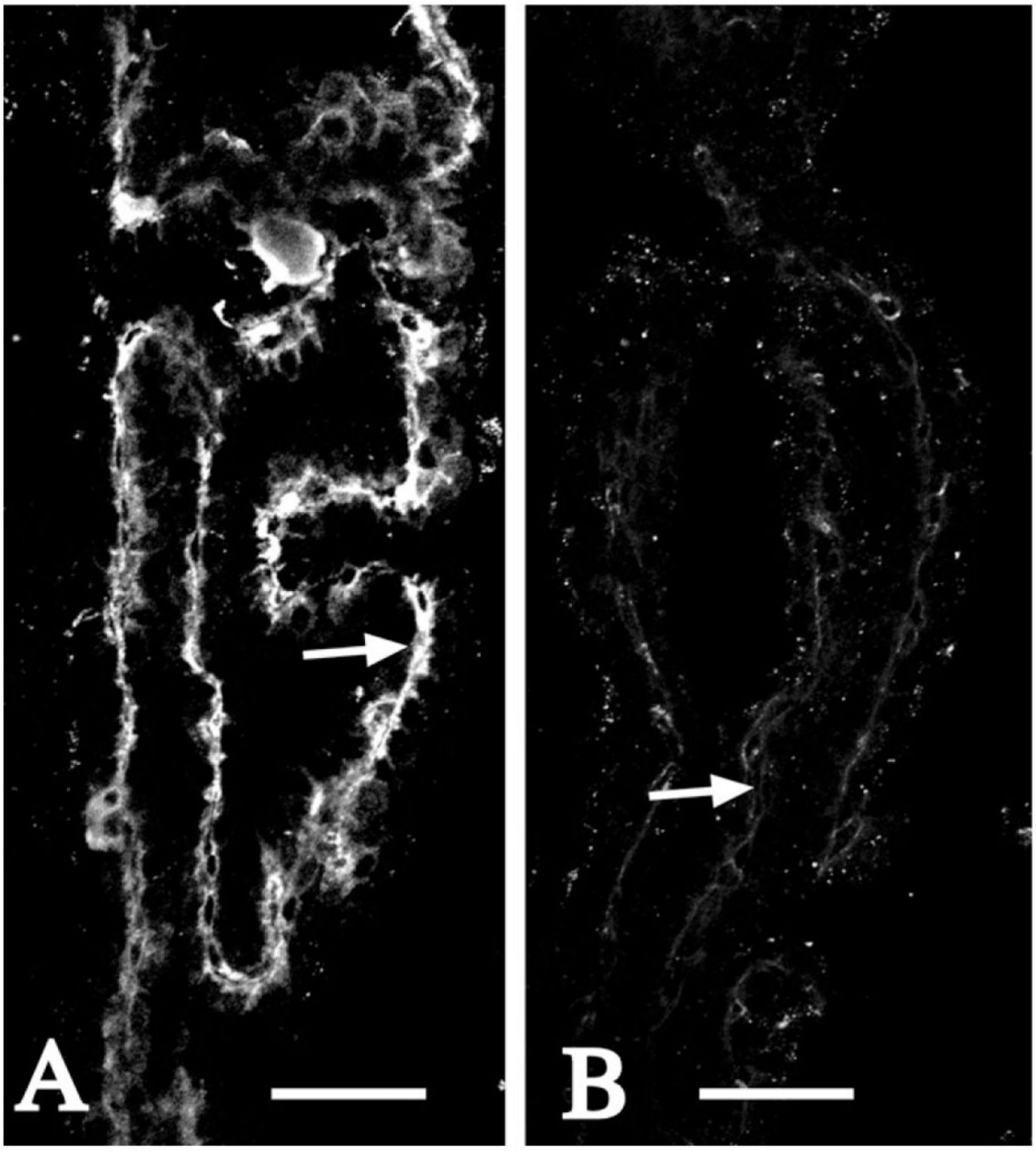

Fig. 8.

Confocal images of immunofluorescence labelling for GLT1b in the choroid plexus (arrows) of a normal (control) mouse (A) and a GLT1 knockout mouse (B). Strong labelling is evident in the control mouse but specific labelling is absent in the knockout mouse. Scale bar: 50 μm.

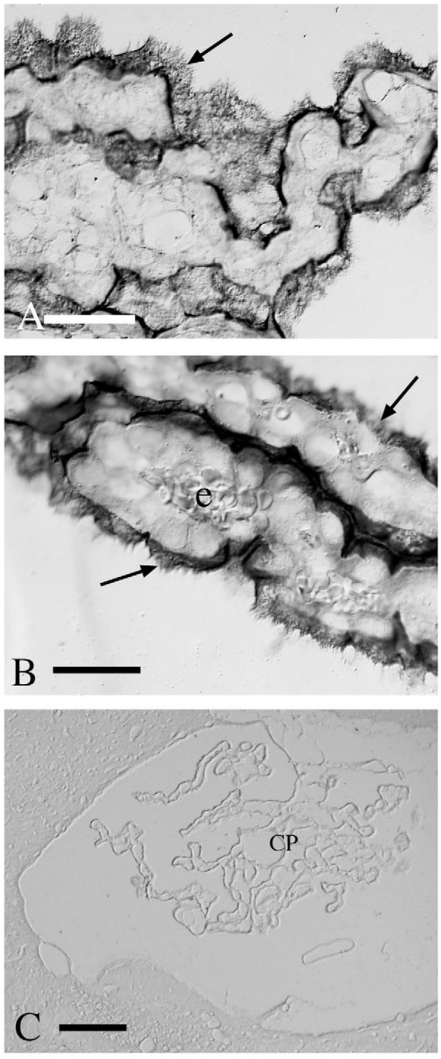

Fig. 9.

Immunocytochemical localisations for CGAP using the antibodies CGAP475 (A) CGAP 12 (B) or pre-immune sera (CGAP475 preimmune) (C). In A and B, immunolabelling is associated with the apical surface of the epithelial cells (arrows). By contrast no labelling of the choroid plexus (CP) was observed with the pre-immune serum (C). Scale bars: (A) and (B) 25 μm, (C) 100 μm.

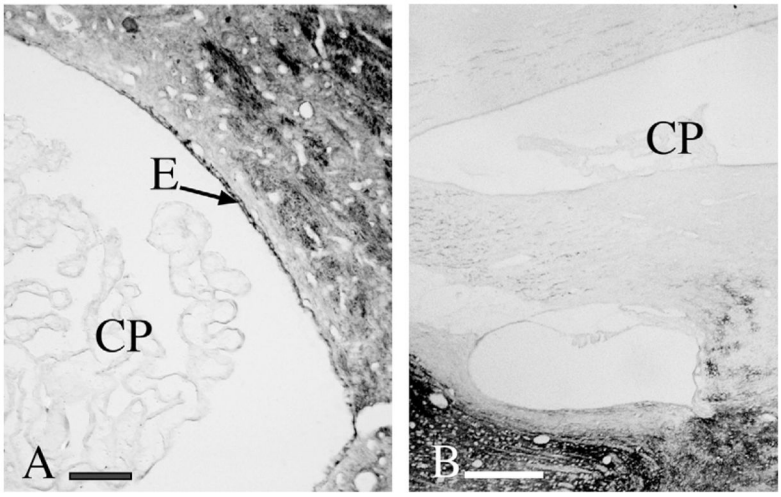

Fig. 10.

Immunolabelling for EAAC1 (A). Labelling is present in the ventricular epithelial cells lining the wall of the fourth ventricle (E, arrow) but absent from the choroid plexus (CP). (B) Labelling for GAT4. Labelling is present in (thalamic) grey matter but absent from the choroid plexus in the lateral ventricles. Scale bars: (A) 50 μm and (B) 100 μm.

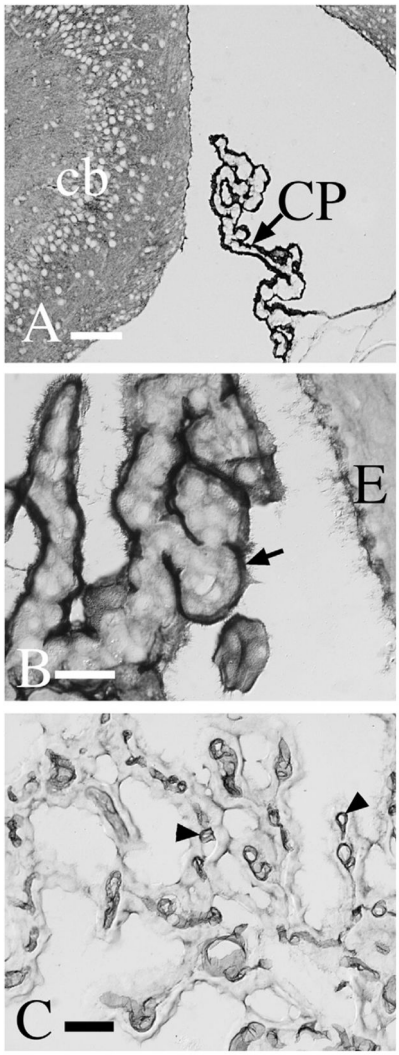

Fig. 11.

(A and B) low and high magnification images of labelling for NHERF1. Labelling is present in the choroid plexus (CP) as well as surrounding brain tissues such as the cerebellum (cb). Labelling is associated with the ventricular epithelium (E) as well as the apical surface of the choroidal epithelium (arrow). (C) depicts labelling for NHERF2, which is expressed by the endothelial cells of the choroid plexus (arrowheads). Scale bars: (A) 50 μm, (B) and (C) 25 μm.

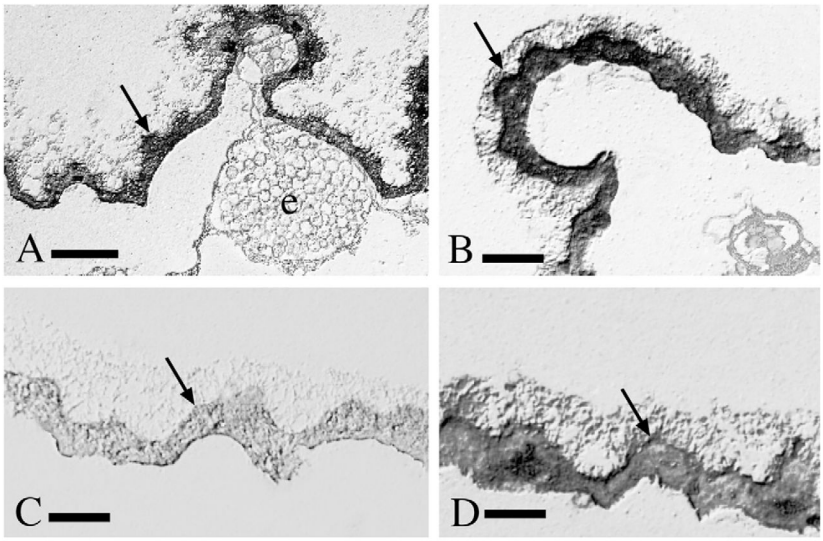

Fig. 12.

(A) Uptake of aminoadipic acid into epithelial cells (arrow) but not other cells such as the erythrocytes (e) in the luminal vessels. (B) uptake of D-aspartate into the epithelial cells (arrows) and in the presence of TBOA (C) or DHK (D). TBOA markedly attenuates uptake whereas DHK has a lesser effect. Scale bars: (A) 25 μm and (B)–(D) 10 μm.

Table 1

Primers used for the identification of EAATs, CGAP and NHERF in the choroid plexus.

mRNA	GenBank accession	Forward oligo sequence	Reverse oligo sequence	Product size (bp)
GLAST	NM_019225.1	ATGACAAAAAGCAACGGAGAAGAGC	TATCAGAGTAGGGAGGAAAGAGGA	1744
GLAST1a	AF265360	CATTGTGGGAATGGCGGC	TATCAGAGTAGGGAGGAAAGAGGA	1432
GLAST1b	AY954110	ATGGAGACTCTGACCCGGATC	GGAGGCGGTCCCTTATTGTTAT	629
GLAST1c	HQ699552	ATGACAAAAAGCAACGGAGAAGAGC	AGGTGCATACCTGATTAAAT	533
GLT1a	X67857.1	GCTCGTCGCCACTGTCTCCA	CATAAGATACGCTGGGGAGTT	1824
GLT1b	AF465909.1	ACTTTGCCTGTCACCTTCCGTTGC	ATGCAGGTCTCGATATCCAGG	594
GLT1c	AY578981.1	ACTTTGCCTGTCACCTTCCGTTGC	TAAACCCACGATTGATATTCCACA	591
GLT1(ex9skip)	X67857.1	GTGACTGTAAGGGATAGAATGAG	CATAAGATACGCTGGGGAGTT	364
GLT1b(ex9skip)	AF465909.1	GTGACTGTAAGGGATAGAATGAG	ATGCAGGTCTCGATATCCAGG	268
EAAC1	NM_013032.3	CGCTCCCCGATTCCTCACAAA	CTTCACAGTCCCAGGCATCTA	1638
EAAT4	NM_032065.1	CGCTGACCCGAGGCTGAG	CCGCCCTGGACAAAGAGCTA	1736
EAAT5	NM_001108973.1	ACAACCTACAGTGCATGGAAT	ACTCCTACGCTTAGCCCAAAC	1763
CGAP	NM_001107673	CTGGGCATGAGAAGGTGGTT	GCAGGAGAGGGCAACAAAGAT	884
NHERF1	NM_021594.1	AGAAGGGTCCGAACGGCTACG	TCCTTGTCTACCACCAGCAGCTTG	641
NHERF2	NM_053811	CCGCTTGTGTCGCCTGGTGC	CAAGGCTGGTCCCGTTCGTG	761

Abbreviations: EAAC, excitatory amino acid carrier; EAAT, excitatory amino acid transporter; GLAST, glutamate aspartate transporter; GLT, glutamate transporter; CGAP, cystine-glutamate antiporter; NHERF, sodium-hydrogen exchanger regulatory factor.

Table 2

Target epitopes for primary antibodies used to detect transporters and related proteins in the choroid plexus.

Antibody	Antigen
GLAST N′	Synthetic peptide, residues 1–24. MTKSNGEEPRMGSRMERFQQGVRK
GLAST C′	Synthetic peptide, residues 503–518. KNRDVEMGNSVIEENE
GLAST1a	Synthetic peptide, residues 55–66. VSAVIVGMAALD
GLAST1b	Synthetic peptide, residues 425–435. QIITIRDRLRT
GLAST1c	Synthetic peptide, residues 170–180. FLDLIRYAPLG
GLT1a	Synthetic peptide, residues 559–573. SADCSVEEEPWKREK
GLT1b	552–562 PFPFLDIETCI
GLT1 × 9	Synthetic peptide residues 424–434. QIVTVRDRMRT
CGAP475	Synthetic peptide residues 475–499. KGGYLQGNVSGRLPSVGDQEPPGHE
CGAP12	Synthetic peptide, residues 12–36. KGGYLQGNMSGRLPSMGDQEPPGQE
NHERF1	Full length recombinant NHERF-1
NHERF2	Carboxyl terminal 106 amino acids of NHERF-2
GAT1	Synthetic peptide, residues 585–599. EQPQAGSSASKEAYI
GAT4	Synthetic peptide, residues 574–585. WKTEGTLPEKLQ