Cell Tissue Res (2004) 315:305–310
DOI 10.1007/s00441-003-0849-3

REGULAR ARTICLE

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Localization and expression of the glutamate transporter, excitatory amino acid transporter 4, within astrocytes of the rat retina

Received: 16 September 2003 / Accepted: 2 December 2003 / Published online: 16 January 2004

Abstract Mechanisms for the removal of glutamate are vital for maintaining normal function of the retina. Five excitatory amino acid transporters have been characterized to date from neuronal tissue, all of which are expressed within the retina except excitatory amino acid transporter 4 (EAAT4). In this study we examined the expression and localization of the glutamate transporter EAAT4 in the rat retina using RT-PCR and immunocytochemistry. RT-PCR using rat EAAT4 specific primers revealed a prominent 296-bp product in the retina, cortex and cerebellum. The identity of the EAAT4 fragment was confirmed by DNA sequencing. We examined the tissue expression levels of EAAT4 in cortex, retina and cerebellum using real-time PCR. The highest expression level was found in the cerebellum. Expression in the cortex was approximately 3.1% that of the cerebellum and the retina was found to have approximately 0.8% the total cerebellar EAAT4 content. In order to examine the specific cell types within the retina that express EAAT4, we performed immunocytochemistry using a rat EAAT4 specific antiserum. Cellular processes within the nerve fibre layer of the retina were intensely labelled for EAAT4. Double labelling EAAT4 with glial fibrillary acidic protein (GFAP) revealed extensive colocalization indicating that EAAT4 is localized within astrocytes within the retina. Double labelling of EAAT4 and the glutamate transporter EAAT1 (GLAST) revealed extensive colocalization suggesting that astrocytes in the retina express at least two types of glutamate transporters. These results suggest that astrocytes within the retina are well placed to provide mechanisms for glutamate removal as well as controlling cellular excitability.

Keywords Glutamate transporter · Retina · EAAT · GLAST · Astrocytes · Müller cell · Rat

This work was supported by grants from the National Health and Medical Research Council (Grant #208950) and Retina Australia.

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Introduction

Glutamate is the main excitatory neurotransmitter in the retina, where it mediates neurotransmission between the major neuronal classes making up the retinal through pathway (photoreceptors, bipolar cells and ganglion cells). In order to maintain normal retinal function, systems for the rapid removal and inactivation of glutamate are necessary. The retina possesses sodium-dependent high affinity uptake systems for glutamate (Ehinger 1977; Brandon and Lam 1983; Fletcher and Kalloniatis 1997).

At the molecular level five excitatory amino acid transporters (EAATs) have been cloned from human tissue: EAAT1–5 (Seal and Amara 1999; Danbolt et al. 1998). Rat homologs of the transporters EAAT1–4 have also been cloned, including EAAT1 (GLAST), EAAT2 (GLT1), EAAT3 (EAAC1) and EAAT4 (Kanai and Hediger 1992; Pines et al.1992; Stork et al. 1992). These transporters demonstrate variable affinity for glutamate, have different chloride conductances and have distinct signalling pathways. In the retina, all the cloned transporters have been demonstrated, with the exception of EAAT4 (Rauen et al. 1996; Eliasof et al. 1998; Rauen et al. 1998).

EAAT4 has been localized primarily within Purkinje cells of the cerebellum (Fairman et al. 1995; Furuta et al. 1997; Nagao et al. 1997; Dehnes et al. 1998). In addition to the cerebellum, expression of this transporter has been observed in other regions of the CNS, such as the cortex, brainstem and hippocampus (Furuta et al. 1997; Massie et

al. 2001). A recent study suggests that EAAT4 may be expressed by astrocytes within some regions of the CNS (Hu et al. 2003). EAAT4 differs from the other glutamate transporters in having a high chloride permeability. Consequently, it has been suggested that EAAT4 may be acting more as a chloride channel in Purkinje cells than as a glutamate transporter.

To date, EAAT4 has not been described in the retina. Rat EAAT4 has recently been sequenced and thus the expression level of EAAT4 within retina using specific rat primers is now possible. The aim of this study was to examine the expression and localization of EAAT4 in the mammalian retina.

Materials and methods

All experiments were performed in accordance with the guidelines for the welfare of experimental animals issued by the Federal Government of Australia and the Society for Neuroscience. All efforts were made to minimize animal suffering and the number of animals used.

Animal and tissue preparation

Rats were deeply anaesthetized with ketamine (60 mg/kg) and sacrificed by anaesthetic overdose with pentobarbital (72 mg/kg). The eyes were enucleated, the anterior segment and vitreous removed, and the posterior eyecup placed in chilled fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. Following fixation, the retinae were dissected from the eyecup, cryoprotected in graded sucroses (10%, 20%, 30%), and either sectioned vertically at 12 μm on a cryostat, or processed free-floating.

For molecular experiments, eyes were removed from the rats, the anterior contents discarded and the retina dissected free from the posterior eyecup. The retinae were then snap frozen in liquid nitrogen and stored at −70°C until use. Other tissues investigated for expression of EAAT4 included cerebellum, liver, kidney, lung and cerebral cortex. All these tissues were dissected from the animals, snap frozen in liquid nitrogen and then stored at −70°C until use.

RNA isolation and RT-PCR

Total RNA was isolated from individual rat tissues (10–30 mg) using the RNeasy mini kit (Qiagen, Chatsworth, CA). To eliminate potential genomic DNA contamination, a DNase treatment was included in the isolation protocol. The purified RNA was quantified at 260 nm on a spectrophotometer (Perkin Elmer Co., Norwalk, CT) and RT-PCR performed on 0.5 μg RNA, using oligo dT primers (Omniscript RT kit; Qiagen). For the subsequent amplification, specific rat EAAT4 primers (5'-tgtcctgagggatttcttcg-3' and 5'-aagaggtacccatggctgtg-3') were designed from the published sequence (accession no. U89608). A commercial PCR reaction mix was used (HotstarTaq; Qiagen) and a touchdown protocol [95°C, 15 min; (94°C, 10 s; 70°C, 30 s; decreasing 1°C/cycle, 72°C, 30 s)×15; (94°C, 10 s; 55°C, 30 s; 72°C, 30 s)×25; 72°C 10 min] run on a PCR Express thermal cycler (Hybaid, Ashford, UK). The identity of the amplified 296-bp product was confirmed by automated DNA sequencing. Finally, the full coding sequence of rat retinal EAAT4 was amplified using oligonucleotide primers (5'-ctgacccgaggctgagac-3' and 5'-gcagcccacagtcagttgt-3') and the 1,796-bp product cloned into the pGEM-Teasy plasmid vector (Promega Co., Madison, WI). The retinal sequence was confirmed by automated sequencing of both strands.

Semi-quantitative real-time PCR

The relative expression levels of EAAT4 were determined for rat cortex, cerebellum and retina using real-time PCR (LightCycler; Roche, Mannheim, Germany). RT-PCR was performed as previously described and ~0.025 μg cDNA added to a commercial reaction mix (FastStart DNA Master SYBR Green I; Roche) containing 3 mM $MgCl_2$ and 0.5 μM of the rat EAAT4 primers. After an initial denaturation (95°C, 10 min), a 40-cycle protocol was used (95°C 0 s, 58°C 10 s, 72°C 15 s) with the fluorescent signal detected after the extension step. Hypoxanthine phosphoryl-ribosyl transferase (HPRT) was used as the housekeeping gene. For the HPRT reactions, human primers (forward: 5'-ggaggccatca-cattgtagc-3'; and reverse: 5'-ccacaatcaagacattctttcc-3') were included and the final $MgCl_2$ concentration was 4 mM. The amplification protocol was similar to that described for EAAT4; however, a 60°C annealing temperature was used and the fluorescence signal detected at 82°C. While the human primers had one base pair mismatch in both oligonucleotides when compared to the rat sequence, a single product of the correct size was amplified. Sequencing of the product showed it to be rat HPRT. All amplifications were performed in triplicate and tissue EAAT4 expression levels were corrected for HPRT content and expressed relative to cerebellum.

Antisera

A polyclonal affinity purified antiserum directed against a 21 amino acid synthetic oligopeptide corresponding to the C-terminus of rat EAAT4 was used to immunolabel EAAT4 (Alpha Diagnostic Int., San Antonia, TX, USA). It was used at a dilution of 1:100. This antiserum has been shown previously to be specific for EAAT4 in tissues taken from several regions of the CNS (Dunlop et al. 1999; Hu et al. 2003). We verified the specificity of the antiserum by Western blotting rat cerebellar membranes. Consistent with the findings of others, the antiserum recognized a prominent band at approximately 69 kDa (data not shown).

Retinal Müller cells were identified by double labelling with a mouse monoclonal antibody directed against glutamine synthetase (diluted 1:50; Chemicon International CA, USA). Astrocytes were identified by double labelling with glial fibrillary acidic protein (GFAP; 1:1,000; Chemicon International CA, USA). In order to examine whether EAAT4 was expressed by cells that expressed other glutamate transporters, double labelling experiments were performed with an antiserum directed against GLAST (1:1,000; Chemicon International CA, USA).

Light-microscopic immunocytochemistry

Immunocytochemical labelling was carried out using the indirect fluorescence method as previously described (Fletcher et al. 2000). Briefly, retinal sections were blocked for 1 h in a solution containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.05% Triton X-100 in PB. The primary antibodies were diluted in 3% NGS, 1% BSA, and 0.05% Triton X-100 in PB, and applied overnight at room temperature. After washing in PB, goat-anti-rabbit IgG conjugated to Alexa TM 594 secondary antibody was applied for 1 h (Molecular Probes, Eugene, OR, US; diluted 1:500).

Microscopic analysis

Sections were examined and imaged with a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany), fitted with a Bio-Rad 1024 confocal illuminating system (Bio-Rad, Sydney, Australia), using ×40, ×63, and ×100 objectives. The appropriate fluorescence filters were used (Alexa TM 594/CY3: excitation −568, emission filter 605/32; Alexa TM 488/FITC: excitation −488, emission filter 522/32) with a krypton/argon laser for differential visualization of

the fluorophores. Red and green fluorescence was scanned and imaged separately and later merged with Adobe Photoshop 7.0 (Adobe Systems, USA).

Results

EAAT4 is expressed in the rat retina

RT-PCR was performed on total RNA isolated from several rat tissues, using specific rat EAAT4 primers. A prominent 296-bp product was amplified from cerebellum as has been demonstrated previously (Fig. 1; Fairman et al. 1995). In addition to cerebellum, a specific EAAT4 message was amplified from frontal cortex and retina. The identity of the EAAT4 fragment amplified from retinal RNA was confirmed by DNA sequencing. Amplifications were also performed using total RNA (instead of cDNA), to control for genomic contamination. Neither the EAAT4 fragment nor any other product was observed in any of the tissue RNA samples (data not shown). The full coding sequence of retinal EAAT4 was amplified by designing specific primers to the 5' and 3' untranslated regions of the rat brain sequence (accession no. U89608). The 1,796-bp product was sequenced and showed a high identity to the human (94%) and rat (99%) brain EAAT4 sequences. In order to estimate tissue expression levels, semi-quantitative analysis was performed using real-time PCR (Fig. 1B). This technique is known to provide an accurate measure of gene expression.The rat HPRT gene was used to correct for mRNA content and results were expressed relative to cerebellum. Cerebellum was found to contain the highest levels of EAAT4. By comparison, the cortex contains ~3.1% and the retina ~0.8% of the total cerebellar EAAT4 content.

EAAT4 is localized to astrocytes in the rat retina

In order to determine what cell types within the retina expressed EAAT4, we performed immunocytochemistry using an antiserum directed against the C-terminus of rat EAAT4. Figure 3A shows a vertical section of rat retina immunolabelled for EAAT4. Processes within the nerve fibre layer were labelled for EAAT4. When viewed as a flat mount, EAAT4 labelled spindle-shaped processes that were confined to the nerve fibre layer and had an intricate association with blood vessels (Fig. 2B). The morphology of these processes is consistent with immunolabelling of astrocytes.

It is often difficult to discern the identity of processes around blood vessels because the processes of Müller cells and astrocytes are found neighbouring one another. Consequently, we examined the immunolabelling pattern of EAAT4 in cross sections of optic nerve, because this is an area devoid of Müller cell processes. Like the retina, glial cells within the optic nerve were found to be immunoreactive for EAAT4 (Fig. 2C).

We confirmed that astrocytes in the retina express EAAT4 by examining colocalization with GFAP, a known marker of astrocytes. As shown in Fig. 3, EAAT4 was found to be colocalized with GFAP, suggesting that EAAT4 labelled astrocytes within the retina.

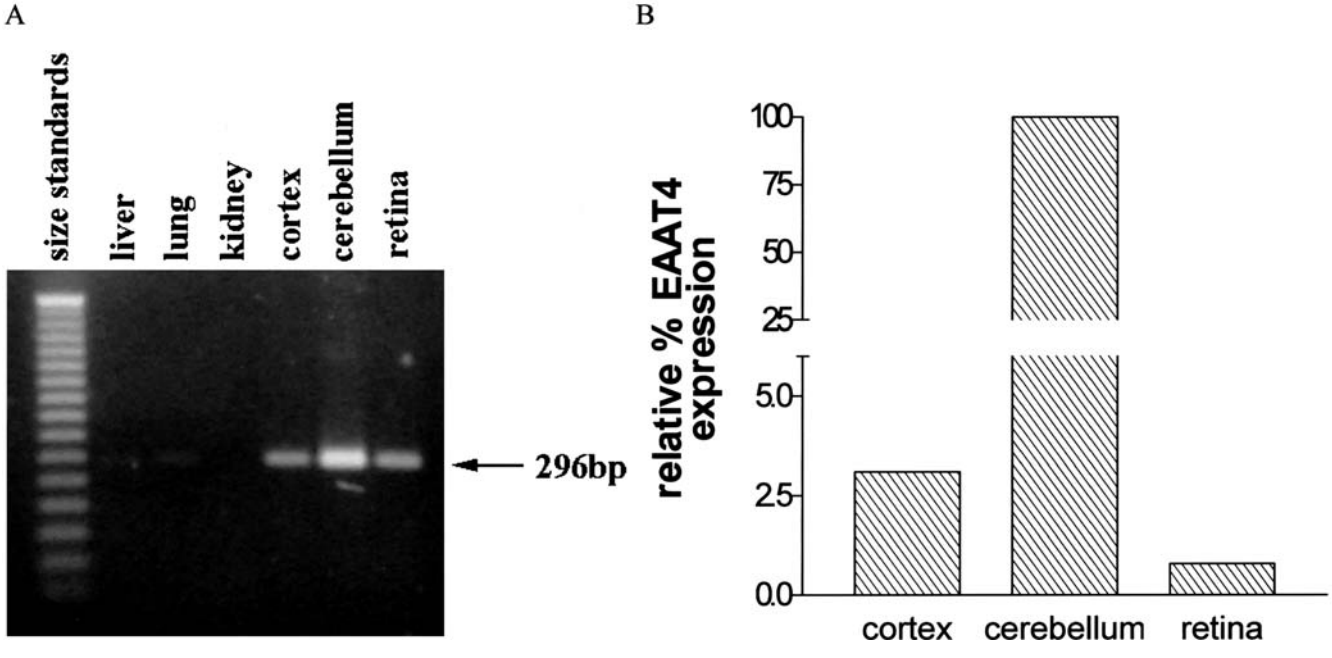

Fig. 1 **A** RT-PCR analysis of EAAT4 expression in rat tissues. Using specific EAAT4 primers designed to the rat sequence, a 296-bp product was amplified in rat cortex, cerebellum and retina. The 50-bp size standards are included as a reference. **B** Semi-quantitative analysis of EAAT4 expression in rat cortex, cerebellum and retina. Results are expressed relative to cerebellum and were corrected using the housekeeping gene HPRT

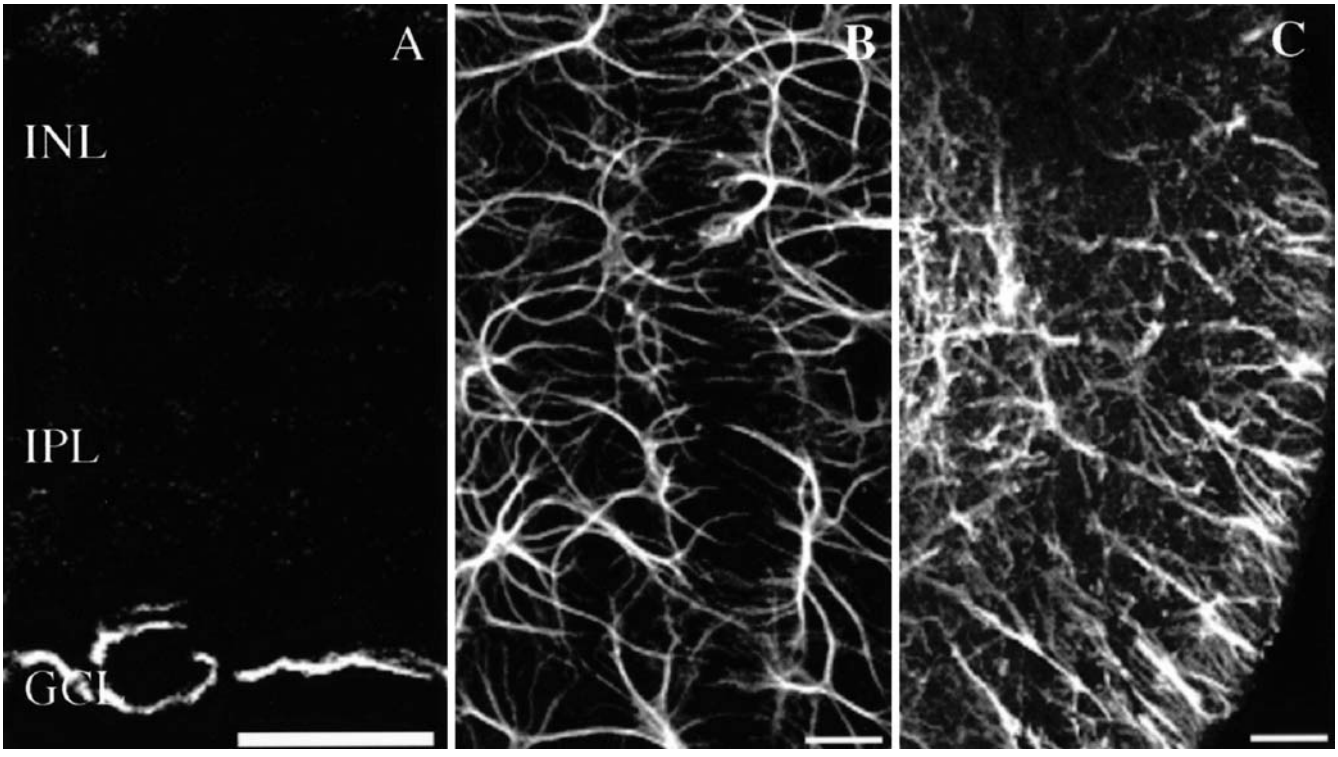

Fig. 2 **A** Vertical cryostat section of rat retina immunolabelled for EAAT4. EAAT4 was localized within astrocytes within the proximal retina. **B** Flat mount of EAAT4 immunolabelled processes within the nerve fibre layer of the retina. Immunoreactive processes can be clearly seen enveloping blood vessels. **C** A cross section of the optic nerve immunolabelled for EAAT4 (*INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer). *Scale bar* 20 μm

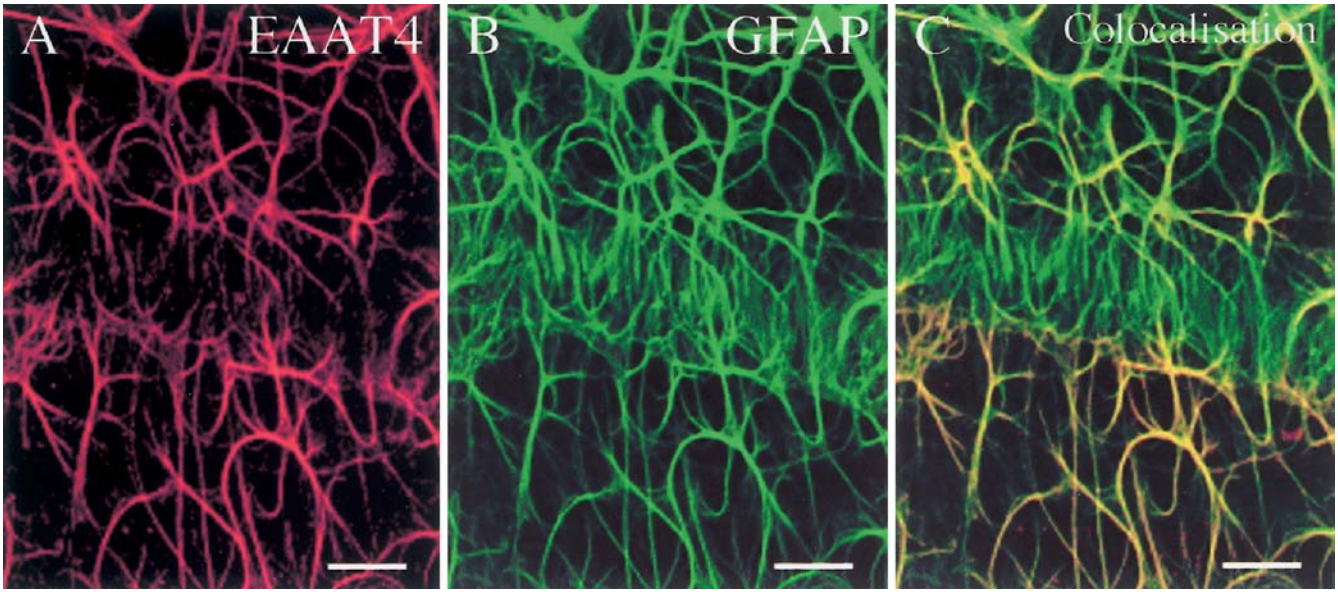

Fig. 3 Flat-mount rat retina double labelled for EAAT4 **(A)** and GFAP **(B)** and areas that colocalize **(C)**. The EAAT4 immunoreactive processes colocalize with GFAP, suggesting that EAAT4 is localized within astrocytes. *Scale bar* 20 μm

Astrocytes within the retina are known to express GLAST. In order to examine whether GLAST and EAAT4 were expressed within the same cells within the retina, we double labelled rat retina with GLAST and EAAT4. As shown in Fig. 4, GLAST and EAAT4 were colocalized within the same cellular processes in the retina, suggesting that EAAT4 was localized within astrocytes in the retina.

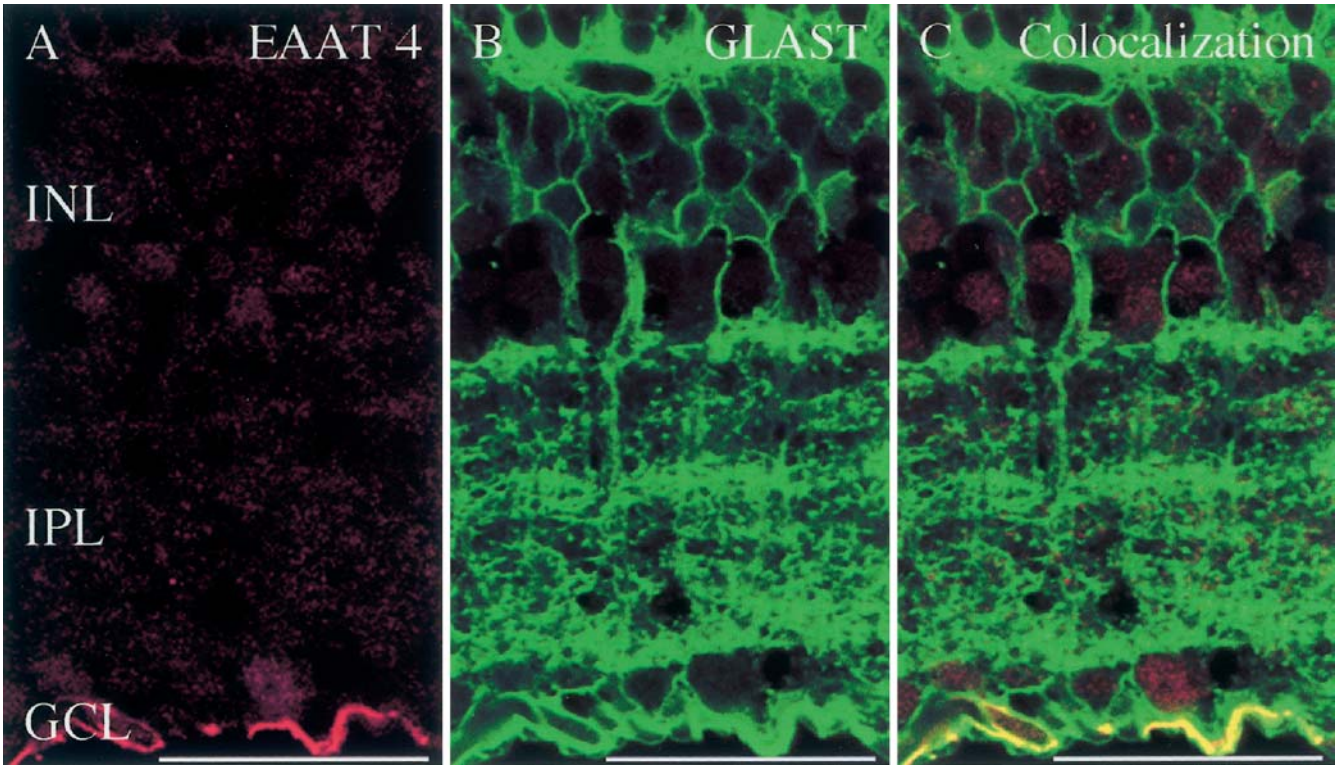

Fig. 4 Immunofluorescent labelling of EAAT4 **(A)** and GLAST **(B)** and areas that colocalize **(C)**. GLAST immunoreactive processes colocalized with EAAT4. Abbreviations as in Fig. 3. *Scale bar* 20 μm

Discussion

Although EAAT4 is known to be expressed within a number of regions of the CNS, it is predominantly associated with glutamate transport by Purkinje cells within the cerebellum (Dehnes et al. 1998; Fairman et al. 1995; Furuta et al. 1997; Nagao et al. 1997; Massie et al. 2001). Its role in glutamate transport within the retina has not been previously described. In this study we provide evidence that EAAT4 is expressed by the retina at significant levels.

This study revealed that EAAT4 was expressed by the retina, in contrast to previous studies (Rauen et al. 1998; Furuta et al. 1997). Rat EAAT4 has only been cloned recently, and a commercially available antiserum directed against rat EAAT4 has only become available in the last few years (Lin et al. 1998). We verified the expression of EAAT4 in the rat retina by sequencing the amplified product, and localizing EAAT4 to astrocytes immunocytochemically.

EAAT4 has been well characterized within Purkinje cells of the cerebellum. Studies examining the expression of EAAT4 within other brain regions have revealed a low level of expression within the frontal cortex, which was confirmed in this study. Indeed, we found that the level of expression of EAAT4 within the forebrain was about 3.1% compared to the cerebellum. Very little is known about the cell types within the neocortex that express EAAT4, although Furuta et al. (1997) suggested that neuronal dendrites were immunolabelled for EAAT4 in the neocortex.

In contrast to the neuronal localization of EAAT4 in the cerebellum, we observed EAAT4 expression within retinal astrocytes. Astrocytes within other regions of the CNS are known to express GLAST and/or GLT1 (Rothstein et al. 1996). In addition, our data confirm previous studies that have shown that Müller cells and retinal astrocytes express GLAST (Rauen et al. 1996; Pow and Barnett 1999).

Our observation that EAAT4 is expressed in astrocytes suggests that these cells express two glutamate transporters. Co-expression of multiple glutamate transporters has been demonstrated previously. EAAC1 and EAAT4 are both expressed by Purkinje cells; each glutamate transporter is thought to mediate glutamate uptake at synapses with two different classes of neurons, namely, climbing fibres and parallel fibres. GLT1 and GLAST are known to be co-expressed within astrocytes in the CNS (Lehre et al. 1995; Chaudhry et al. 1995). Furthermore, ultrastructural studies indicate that GLT1 and GLAST are expressed concomitantly, but to different extents in astrocytes in different regions of the brain (Lehre et al. 1995; Chaudhry et al. 1995). In the retina, astrocytes are found exclusively in the nerve fibre layer. Zahs and Wu (2001) demonstrated morphologically that there are at least two subclasses of astrocyte within the retina: intervascular astrocytes, which are located between the major arterioles, and vascular astrocytes, which have an

intricate relationship with the retinal vasculature. Our data suggest that both these classes of astrocyte express EAAT4. Further ultrastructural studies are required to determine whether EAAT4 and GLAST are localized to different compartments within astrocytes.

Expression of two glutamate transporters within astrocytes may relate to their distinct pharmacological profiles. In contrast to GLAST, EAAT4 is known to have a high chloride conductance. The function of the glutamate-induced chloride conductance of EAAT4 is not known, although it has been postulated that it might be important in controlling neuronal excitability. With the co-expression of GLAST and EAAT4, retinal astrocytes would have a high capacity for removal of synaptically released glutamate as well as mechanisms for regulating cellular excitability.

A major question that arises from this study is the functional significance of the expression of glutamate transporters in retinal astrocytes. Astrocytes are confined to those retinae that have an inner retinal blood supply and have an intricate relationship with retinal blood vessels (Zahs and Wu 2001). They are thought to be important for development of the retinal vasculature and maintaining the blood retinal barrier. Their role in glutamate removal in the retina has received limited attention. In support of a role of astrocytes in glutamate degradation is the expression of glutamine synthetase, the major enzyme involved in glutamate degradation, by retinal astrocytes. Formation of glutamine from glutamate is also important for removal of ammonia. Because of their intricate relationship with retinal blood vessels, astrocytes may provide a mechanism for removal of ammonia from the retina.

Glutamate uptake by retinal astrocytes could be important in regulating communication within the nerve fibre layer and for protecting ganglion cell axons from glutamate induced excitotoxicity. Further work is required to elucidate the precise role of glutamate transporters within astrocytes, and the role that EAAT4 plays in the mammalian retina.

Acknowledgements The authors are very grateful for the assistance of Mr. Guido Tomisich for technical assistance.

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