Excitatory Amino Acid Transporter 5 is widely expressed in peripheral tissues

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Abstract

It is routinely stated in the literature that Excitatory Amino Acid Transporter 5 (EAAT5) is a retina-specific glutamate transporter. EAAT5 is expressed by retinal photoreceptors and bipolar cells, where it serves as a slow transporter and as an inhibitory glutamate receptor, the latter role is due to the gating of a large chloride conductance. The dogma of an exclusively retinal distribution has arisen because Northern blot analyses have previously shown only modest hybridisation in non-retinal tissues. Others have re-interpreted this as indicating that EAAT5 was only present in retinal tissues. However, this view appears to be erroneous; recent evidence demonstrating abundant expression of EAAT5 in rat testis prompted us to re-examine this dogma. A new antibody was developed to an intracellular loop region of rat EAAT5. This new tool, in concert with RT-PCR and sequencing, demonstrated that EAAT5 is widely distributed at the mRNA and protein levels in many non-nervous tissues including liver, kidney, intestine, heart, lung, and skeletal muscle. We conclude that EAAT5 is a widely distributed protein. Whether it functions in all locations as a glutamate transporter, or mainly as a glutamate-gated chloride conductance, remains to be determined.

Introduction

High affinity sodium-dependent glutamate transporters play a central role in the homeostasis of extracellular glutamate concentrations in tissues such as the central nervous system, where glutamate is used as a neurotransmitter and must therefore be tightly regulated.[1] In the nervous system a variety of glutamate transporters are utilised. The five key types are Excitatory Amino Acid Transporter 1 (EAAT1; also called GLAST), EAAT 2 (also called GLT-1), EAAT3 (also called EAAC1), EAAT4 and EAAT5. Each of these types in turn probably exists as multiple splice variants.[2] EAAT5, which was cloned by Arizza *et al*.,[3] is the least studied of all the EAATs, possibly because of the widely stated view that it is a retina-specific glutamate transporter, and thus not implicated in glutamate homeostasis in other tissues. This view has been promulgated by our team[4] as well as many others but seems, in retrospect, to reflect (at least from the perspective of our own team), from a misinterpretation of the findings of Arizza *et al*.[3] In their study, strong hybridisation signals were observed in Northern blots of human retina, but less intense signals were evident in other tissues, with some bands at different sizes. Whilst the significance of these bands was briefly discussed as possibly representing splice variants, the overall focus of the discussion on the retina appears to have lead to the assumption that this meant it was a retina-specific glutamate transporter. Aside from the misinterpretations of the original findings of Arizza *et al*.[3] by ourselves and others, technical issues may have also influenced the results of the study. These technical issues may have included parameters such as the quality of the mRNA used to generate the original human cDNA library from which EAAT5 was cloned. Retinal cDNA is relatively easy to source with minimal degradation (as donor human eyes are harvested rapidly for corneal donation), when compared to other tissues such as brain, where *post-mortem* delays tend to be greater. Perhaps more significantly, Northern hybridisation is very sensitive to alternate-splicing of RNA; changes in overall sequence significantly reduce the hybridisation efficiency. This may influence subsequent interpretations as to the presence or absence of mRNA encoding a specific gene. Our team have now identified multiple splice variants of EAAT5[5] (Genbank accession JF422064, JF422065, JF422066, JF422067, JF422068). Accordingly, the Northern hybridisation studies may have under-represented the overall abundance and tissue distribution of EAAT5 variants. Indeed, Ochiai *et al*. recently showed expression of EAAT5 message and protein in the canine cerebellum and lens tissue,[6] whilst EAAT5 expression has been demonstrated in the vestibular system.[7,8] The final catalyst for our re-evaluation was our recent finding that EAAT5 was abundantly expressed in the testis where it may have a role in directing sperm motility.[9] EAAT5 is particularly interesting since this protein exhibits a relatively large chloride conductance, associated with modest transport activity, suggesting a function more closely related to ligand gated chloride channels than classic transporters. This has lead to the finding that wild type EAAT5 functions as an *inhibitory* presynaptic glutamate receptor in retinal bipolar cells.[10,11] Thus, the chloride conductance properties of EAAT5 may be more important than the transport function. In this study, we have re-evaluated the dogma that EAAT5 is a retina-specific glutamate transporter, using PCR, immunocytochemistry and Western blotting. One key issue, which has become increasingly apparent in the glutamate transporter literature, is that the carboxyl and amino termini of transporters may become inaccessible to antibodies either because of modification of the protein or the cleavage off of such terminal regions.[12,13] This possibility has been raised with respect to EAAT5 to explain the abrupt loss of immunoreactivity for amino and carboxyl terminal regions of EAAT5 as it was transported out of the cell bodies of the retinal bipolar neurons.[4] To counter this possibility, a new antibody was generated in this study against an intracellular epitope of EAAT5 corresponding to a region encoded by exon 6 of the EAAT5 gene. This was chosen because all of the EAAT5 splice variants we have cloned retained exon 6. Accordingly, it was deemed that an antibody against this region would potentially detect all known forms of EAAT5. Moreover, it was considered probable that this intracellular epitope in the middle of the protein was unlikely to be eliminated by cleavage events under normal physiological circumstances.

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Materials and 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.

RT-PCR screening of rodent tissues for EAAT5

Total RNA was isolated from multiple tissues of Dark Agouti rats that had been euthenased by an overdose of sodium pentobarbital (100 mg/Kg, IP). Tissues investigated included retina, liver, kidney, small and large intestine, heart, lung, pancreas, and skeletal muscle. RNA was isolated using TriZol® reagent (Invitrogen, Carlsbad, CA, USA) following precisely the manufacturer's instructions. 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, 0.15 µM EAAT5 primers, 0.05 µM β-actin primers, 1.5 mM $MgCl_2$, and 2.5U BIOTAQ DNA polymerase (Bioline Pty Ltd, Alexandria, NSW, Australia) in 1xPCR buffer. PCRs were performed using the following conditions: initial denaturation at 95ºC for 2 min followed by 30 cycles of amplification (95ºC for 30 s, 60-62ºC for 30 s and 72ºC for 40 s). The reaction products were separated on 2% agarose gels and visualized by staining with 0.5 µg/mL ethidium bromide (Sigma-Aldrich). Sequences of sense and antisense primers were: F-rEAAT5 (1200) 5'-ACCAGGAAGAACCCCATCGTC-3' and R-rEAAT5 (1643) 5'-CGCATCGCCCAGCACGTTAATC-3' derived from the GenBank sequence NM_001108973.1 corresponding to rat EAAT5; F-β-actin (369) 5'-GTTTGAGACCTTCAACACCCCAG-3' and R-β-actin (1080) 5'-CTGCTTGCTGATCCACATCTGC-3' derived from GenBank sequence NM_031144.3 corresponding to rat β-actin. A blank control (water instead of cDNA template) was used as a negative control in all PCR experiments to confirm that nonspecific PCR amplification is not occurring. Preliminary optimization of the conditions showed that co-amplification of EAAT5 and β-actin transcripts was occurring linearly through cycles 25-30. Fluorescence from ethidium bromide staining of each EAAT5 signal was compared with that of β-actin, by calculating the ratio (fluorescence units of EAAT5/fluorescence units of β-actin); densitometric analysis was performed using NIH Image J software.

Cloning of EAAT5

To confirm the veracity of our PCR data, EAAT5 was cloned from several representative tissues (retina, kidney, lung and small intestine). PCR was performed using KOD Hot Start DNA Polymerase (Toyobo, Osaka, Japan) and primers [F-rEAAT5 (1200) and R-rEAAT5 (1643)]. The PCR mixture (50 mL) contained: 2 µL cDNA, 200 µM dNTPs, 0.2 µM each of sense and antisense primers, 1 mM $MgSO_4$, and 2U of KOD Hot Start DNA polymerase in 1X PCR buffer. Polymerase activation was at 95°C for 2 min followed by 30 cycles of amplification (95°C for 20 s, 60°C for 15 s, 70°C for 40 s). The reaction products were separated on a 1.5% agarose gel and visualized by staining with 0.5 µg/mL ethidium bromide (Sigma-Aldrich, Castle Hill NSW, Australia). The PCR products were excised from the gel, purified using a Gel extraction kit (Qiagen Pty Ltd., Doncaster VIC, Australia) and subcloned into the pCR Blunt II-Topo vector (Invitrogen) according to the manufacturer's instructions and sequenced in both directions by the Australian Genome Research Facility (Brisbane, Qld, Australia).

Antibodies

A rabbit was immunized according to standard protocols,[14,15] using a peptide with the unique amino acid sequence TPLVSFCQCLNESV, corresponding to EAAT5 amino acid residues 254-267 (Genbank accession NP_001102443.1). This forms part of an intracellular loop encoded by exon 6 of the EAAT5 gene, which is conserved in all the forms of EAAT5 that we have cloned to date. Immunisation was conducted using our standard protocols.[14,16] Sera were tested by dot blotting[14] using conjugates of peptides coupled with bovine serum albumin. The immunizing peptide and controls (irrelevant synthetic peptides corresponding to N and C-terminal regions of EAAT5 respectively) were examined. One microliter of conjugate was applied to nitrocellulose membranes (Pall, Cheltenham Victoria, Australia) and probed with the primary antisera or preimmune sera at dilutions of 1:500-1:50,000. Detection was revealed using a biotinylated anti-rabbit secondary antibody and streptavidin-horseradish peroxidase complex (GE Healthcare, Castle Hill, Australia), with 3,3'-diaminobenzidine (DAB) as a chromogen. Controls included the use of pre-absorption controls and the use of pre-immune sera. Further details are included in the methods sections below.

Lysate preparation and Western blotting

Tissues were rapidly isolated from adult rats that had been euthanized by an overdose of sodium pentobarbital (100 mg/kg, by intraperitoneal injection). Tissues (liver, kidney, lung, skeletal muscle, heart, small intestine, and retina) were 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 (20-50 µg) was dissolved in SDS sample buffer, separated on a 7% SDS polyacrylamide gel and then transferred to nitrocellulose membrane (Pall) 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 in fresh blocking buffer containing primary antibodies overnight at 4ºC. Following four washes with Tris-NaCl-Tween buffer, blots were 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 the EAAT5 antisera. The peptide was added to the diluted antibody and incubated in a refrigerator at 4ºC for 6 h, prior to use.

Cell culture and transfections

The HEK293 cell line was maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and incubated at 37°C in 5% CO_2. Cells were plated into T-25 cm^2 flasks and at ~80% confluency were transfected with pcDNA3:EAAT5, pBK-CMV:GLT-1a or pBK-CMV:GLAST using X-tremeGENE HP (Roche) following the manufacturer's instructions. Three to four days after transfection, cells were rinsed with ice-cold PBS and harvested in lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche). After lysis for 60 min at 4°C, lysates were clarified by centrifugation for 20 min at 17,000× *g* at 4°C. Lysates (20-50 µg) were used in Western blotting as described above.

Immunocytochemistry

Immunoperoxidase labeling for EAAT5 was performed as previously described using standard methods[14] on paraffin-wax embedded sections of rat tissues. Briefly, rat tissues were fixed by perfusion 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 micro-

scope slides. Sections were de-waxed with xylene and rehydrated through a graded series of ethanol/water solutions. 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 (GE Healthcare) and streptavidin-biotin-horseradish peroxidase conjugates (GE Healthcare) 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. Preabsorption of antisera was always used to confirm the specificity of such.

Microscopy

Bright field imaging was performed using a Nikon 80i equipped with an Olympus DP70 camera. All images were imported into Adobe Photoshop for minor brightness and contrast adjustments prior to composition of plates using Adobe Freehand.

Results

EAAT5 mRNA expression in multiple tissues

Expression of EAAT5 mRNA in various rat tissues was determined by RT-PCR (Figure 1A). A dominant EAAT5 amplification product of 443-bp (corresponding to exons 7-10) was obtained in mRNA samples from retina, small intestine, large intestine, heart and lung. Lower levels of EAAT5 mRNA expression were observed in liver, kidney, pancreas and muscle. By normalizing the EAAT5 mRNA signal to that of β-actin, the relative abundance of EAAT5 in small and large intestine, heart and lung were determined to be approximately 70-80% as that of retina (Figure 1B). By comparison, levels of EAAT5 mRNA in liver, kidney, pancreas and muscle were approximately 20% of that in retina (Figure 1B). The identities of the 443-bp amplification products from several representative tissues [retina (control), kidney, lung and small intestine] were confirmed by cloning and sequencing, which revealed a 100% sequence identity for each with the published rat EAAT5 sequence (NM_001108973.1) (*data not shown*). RT-PCR was also performed using another primer combination (F988 and R1643), which produced an amplification product of 655-bp (corresponding to exons 6-10 of rat EAAT5) in retina (control) and all tissues examined (liver, kidney, pancreas, small and large intestine, heart, lung and skeletal muscle) (supplementary Figure 1). The identities of the 655-bp amplicons in several representative tissues [retina (control), lung and kidney] were subsequently confirmed by direct cloning and sequencing. Other primer combinations (*e.g.*, F1139 and R1594 corresponding to exons 7 to 9) were also used to validate expression of EAAT5 mRNA in retina and all tissues examined (*data not shown*).

Specificity of the exon-6-directed EAAT5 Antibody

The polyclonal antibody used in the present study was raised against a peptide corresponding to an intracellular loop region of EAAT5 (encoded by exon 6) (Figure 2A) with very little homology to other EAAT members (Figure 2B). Specificity of the new EAAT5 antibody for its tar-

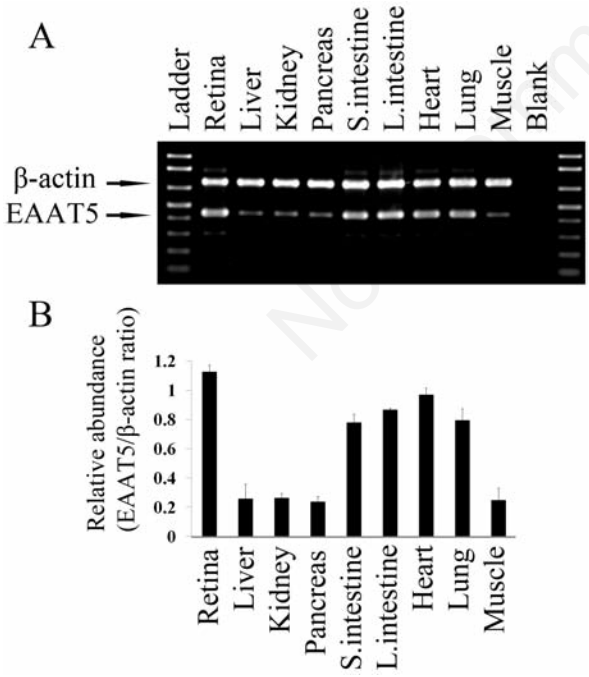

Figure 1. Tissue distribution of EAAT5 mRNA. A) RNA from various rat tissues (as indicated) were reverse transcribed and used in PCR with EAAT5 (lower bands) and β-actin (upper bands) primers. An aliquot of each PCR was electrophoresed on a 2% agarose gel and visualised with ethidium bromide staining. A water control (blank) is shown. B) Densitometric analysis of EAAT5 amplification products from three separate experiments; data are shown as mean ± SD.

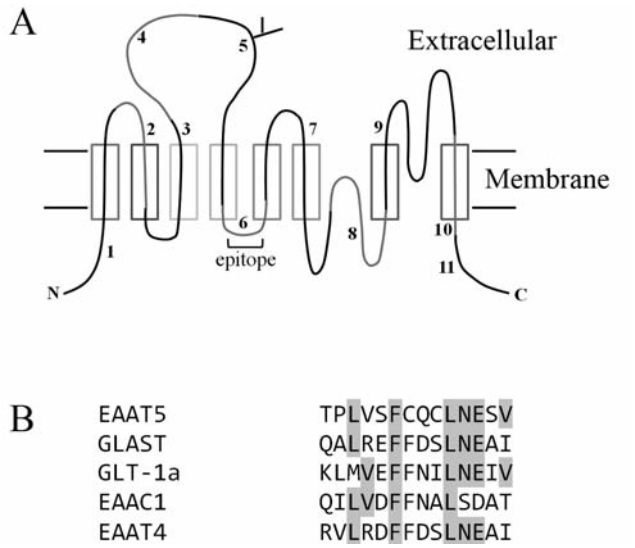

Figure 2. The EAAT5 loop epitope. A) Schematic representation of wild-type EAAT5 secondary structure with exon boundaries overlayed onto the predicted topology; an antibody was generated against an intracellular epitope of EAAT5 (as indicated) corresponding to a region encoded by exon 6 of the EAAT5 gene. B) Alignment of the EAAT5 epitope sequence against that of other EAAT members (GLAST, GLT-1a, EAAC1 and EAAT4); identical residues are highlighted in grey.

get was initially verified using dot blots of the immunizing peptide. The antiserum specifically detected the target peptide but not irrelevant peptides (Figure 3A). To further assess antibody specificity, HEK293 cells were transfected with a vector encoding wild-type EAAT5 as well as vectors encoding GLAST (EAAT1) and GLT-1a (EAAT2) as controls. Western blotting was performed on cell extracts from the transfected cells to detect specificity of antibody-antigen interaction. The EAAT5 loop antibody detected a single band migrating at approximately 61-62 kDa in lysates of cells transfected with EAAT5 but not in lysates of cells transfected with GLAST or GLT-1a (Figure 3B). The relative mass of the EAAT5 immunoreactive band corresponded to the predicted molecular weight of EAAT5 (61,340.23 Da; predicted by ExPASY Bioinformatic Resource Portal). Pre-absorption of the antiserum resulted in no detectable labelling (data not shown). An antibody directed against the C-terminal region of EAAT5 also yielded similar results, demonstrating that the EAAT5 loop antibody was in fact detecting the correct target protein in transfected cells (Figure 3B).

Tissue distribution of EAAT5

Western blot analysis showed that EAAT5 was identified in protein extracts of all tissues examined (Figure 4). In retina (control), a band at approximately 62 kDa was evident (corresponding to wild-type EAAT5) along with smaller sized bands of ~57 to ~43 kDa that corresponded to the sizes of the splice variants of EAAT5 that have been recently identified.[5] Each of the other tissues examined showed expression of a band at around 62 kDa, presumably corresponding to wild-type EAAT5. Additional smaller sized bands were detected in liver, kidney and heart, which may be indicative of differential splicing of EAAT5 in such tissues.

Immunolocalization of EAAT5

Each of the tissues chosen for imunocytochemical investigation displayed immunoreactivity for EAAT5. In the retina (Figure 5A), EAAT5 immunoreactivity was associated with the previously described locations for EAAT5, labelling being notable in photoreceptors including their terminals in the outer plexiform layer (Figure 5B), in bipolar cells and in ganglion cells.[4,17] The kidney exhibited strong labelling in the proximal tubules, labelling accordingly being evident in the outer stripe of the cortex, with weaker labelling in the inner stripe and no labelling in the medullary region (Figure 5C). In the gut, strong labelling was evident in structures such as the goblet cells of the small intestine (Figure 5D). Similarly, consistent labelling was also evident in the lung; punctate labelling appeared to be associated with the membranes of epithelial cells that form the alveoli (Figure 5E). In skeletal and heart muscle labelling was also evident, such labelling being localised around the individual muscle fibres (Figure 5F,G).

Discussion

In the present study, EAAT5 mRNA and protein was demonstrated in multiple peripheral tissues in the mammalian body, in addition to the known localisation in retina. Subsequent sequencing data, using lung, kidney and small intestine as representative peripheral tissues, showed that the larger amplicons detected in such tissues corresponded to EAAT5. Our new antibody detected multiple bands in several tissues including the retina. This observation is compatible with our prior observations that EAAT5 can exist as multiple differentially spliced isoforms[5] (see Genbank accession #JF422064, JF422065, JF422066, JF422067, JF422068), with a range of predicted translated molecular weights between 43 kDa and 57 kDa. The consistency between the molecular data and the protein data in this study strongly supports the view that the tissues investigated express EAAT5. These observations are, however, in apparent contradiction to the commonly articulated dogma that EAAT5 is a retina-specific glutamate transporter. It is plausible however that the dogma has arisen primarily as a response to a lack of evidence; accordingly in the light of the current evidence, the dogma should be revised to indicate that EAAT5 is widely expressed. This view is supported by the recent findings of EAAT5 in the anterior segment of the eye,[6] cerebellum,[6] testis[9] and vestibular system.[7,8]

Our findings now evoke questions as to the relevance of EAAT5 in the tissues that express such. The two properties that are central to the known function of EAAT5 in the retina (namely the slow transport of glutamate and the gating of a chloride conductance which may cause hyperpolarisation of the cells expressing EAAT5) are presumably also evident in other cells in other locations. Skeletal and heart muscle both contain high concentrations of glutamate, and at least in the hypoxic heart, it

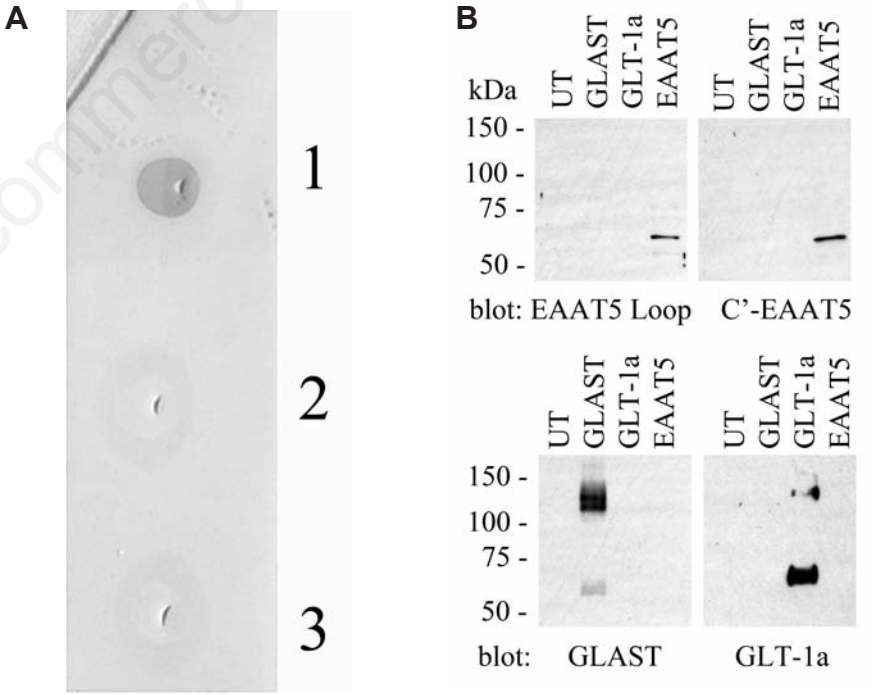

Figure 3. Characterization of EAAT5 loop antibody. (A) Dot blot showing the antibody against an epitope encoded by exon 6 of EAAT5 selectively detected the immunising peptide (1) but not other irrelevant peptides (2 and 3). (B) HEK293 cells were transfected with plasmids encoding GLAST, GLT-1a or EAAT5. Cell extracts were resolved by SDS-PAGE and immunoblotted with the EAAT5 loop antibody, an antibody directed the C-terminal portion of EAAT5 (C'-EAAT5), or antibodies directed against GLAST or GLT-1a. Both the EAAT5 loop antibody and C'-EAAT5 antibody detected a band of ~62 kDa in EAAT5 transfected cells but not in untransfected cells (UT) nor in cells transfected with GLAST or GLT-1a.

is known to be released in response to stimuli such as hypoxia, and that one mechanism by which this might be mediated is by the reversal of sodium-dependent glutamate transport.[18] There appears to be an association between glutamate transport and exercise in skeletal muscles,[19,20] with fluxes of glutamate, both out of, and into the muscles. If EAAT5 mediates glutamate uptake under such exercise conditions then it is plausible that release of glutamate from cells that are actively contracting could evoke a hyperpolarising response that feeds back to modify the contractile event. In the gut, it is known that multiple glutamate transporters may be present though their localisation is not well understood[21] and their roles have not been established beyond the possibility of removal of glutamate from the lumen of the gut as part of ongoing digestive processes. Whether high luminal concentrations of glutamate generated by the digestion of protein cause hyperpolarisation of gut cells, such as the goblet cells and thus influence their secretory activity, is unclear.

The general roles of glutamate transporters in the kidney are unclear. EAAT3 has previously been identified in the kidney using in situ hybridisation studies,[22] whilst Northern analyses have suggested the possible additional expression of GLT-1.[23] However, their roles have not been clearly understood, beyond the view that these transporters provide substrates for a variety of transamination reactions. A close analysis of the literature reveals that EAAT5 has been detected in gene array studies of kidney but never commented upon,[24] but again supports the proposed view that EAAT5 is widely expressed by mammalian tissues. The presence of EAAT5 in what appears to be the proximal tubules (based upon morphological criteria) suggests that EAAT5 may be implicated in reabsorption events. Whether fluxes of glutamate are the intended consequences of EAAT5 expression or chloride fluxes, or a combination of both is unclear. In the lung, the widespread expression of EAAT5 is associated with the transport epithelia. The lung utilises glutamate in a variety of ways, including uptake of such to synthesise glutamine[25] as well as glutathione. Whilst much of the uptake of glutamate appears to be mediated via the cystine-glutamate antiporter, around 10% is via a sodium-dependent transporter;[26] our data would suggest that this sodium-dependent transporter could be EAAT5. Many tissues also express glutamate receptors including heart, kidney, lungs and testis, and such receptors may mediate cell-to-cell communication.[27] It is plausible that EAAT5 may be used as a glutamate transporter in such tissues to reduce extracellular levels of glutamate to levels where these receptors can function appropriately. It has previously been suggested that EAAT5 might serve to regulate sperm motility in the testis, possibly also generating directional cues for the sperm by virtue of the polarised expression of EAAT5 on the heads of sperm and the gradient of glutamate that exists in the reproductive tract.[9] Other comparable hypothetical scenarios can be invoked to explain the functional significance of the

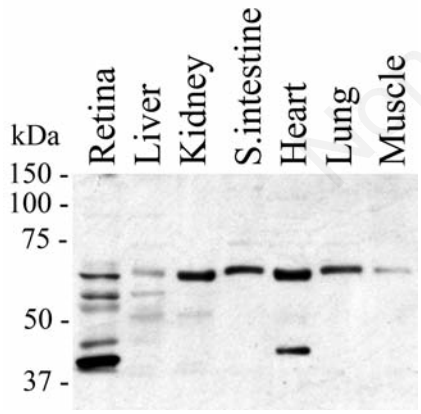

Figure 4. Western blot demonstrating expression of EAAT5 in various rat tissues (as indicated). A ~62 kDa band (corresponding to wild-type EAAT5) is labelled in retina (control) and all other tissues examined. Additional smaller bands (presumed to represent tissue-specific alternate splicing of EAAT5) are also evident in retina, liver, kidney and heart. All lanes were loaded with ~20 µg of total lysate with exception of kidney (~60 µg input).

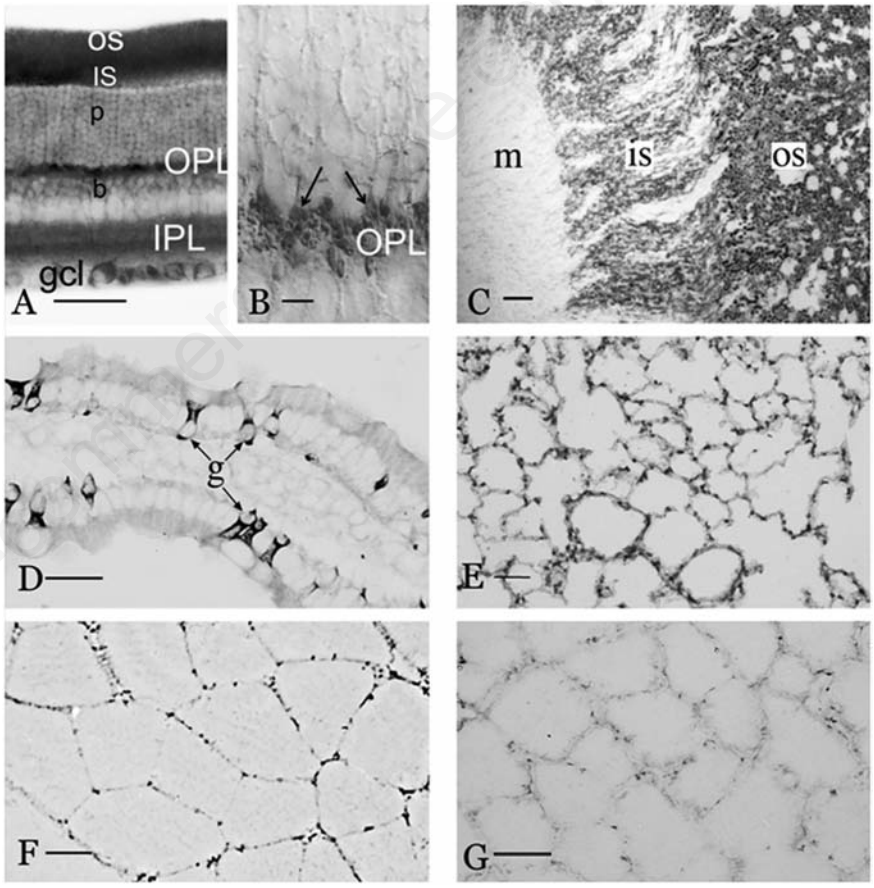

Figure 5. Immunolabelling for EAAT5 in retina (A and B), kidney (C), small intestine (D), lung (E), skeletal muscle (F) and heart (G). In retina (A) labelling is associated with somata of photoreceptors (p) as well as their outer segments (os) and inner segments (is). Strong labelling is associated with the synaptic terminals of the photoreceptors in the outer plexiform layer (OPL). Labelling is similarly evident in somata of bipolar cells (b) and processes in the inner plexiform layer (IPL). Ganglion cell somata in the ganglion cell layer (gcl) are also labelled. (B) arrow indicates labelling of photoreceptor synaptic terminals. In kidney (C), heaviest labelling is in the outer stripe (os) of the cortex, with lesser labelling in the inner stripe (is); labelling was absent from the medulla (m). In the intestine (D), labelling was associated with goblet cells (g) whilst in lung (E), labelling was associated with epithelia of the alveoli. In skeletal muscle (F) and heart (G), labelling was around the periphery of muscle fibres. Scale bars: A, C, F, G, 25 µm; B, 500 µm; D, 50 µm; E, 10 µm.

expression of EAAT5 in these and other tissues such as liver. In all instances these hypothetical scenarios require experimental validation. Many of the roles of EAAT5 will presumably be clarified in due course as a validated EAAT5 knockout mouse becomes available, but such animals are not yet currently available.

In this study the dominant PCR amplicon for mRNA from kidney, lung and small intestine was cloned and sequenced. In each case the amplicon was demonstrated to represent EAAT5. Similar analyses have not as yet been performed on the less abundant and smaller mRNA bands that are present in some of the tissues, but we presume such to represent some of the smaller splice variants we have previously cloned from the retina. There is currently no available data as to whether the splice variants are functional transporters, whether they represent functionally inactive proteins or whether they serve more complex roles. Glutamate transporters that have been studied to date appear to form trimeric complexes with other glutamate transporter molecules. The possibility exists that the formation of heterotrimers containing wild type (full length) EAAT5 and alternately spliced forms could result in modification of the trafficking and function of the resultant heterotrimers.[2,28] A more extensive analysis of the range of EAAT5 splice variants and their expression at the mRNA and protein levels using splice-specific antibodies is the subject of another forthcoming manuscript.

Conclusions

EAAT5 is present in multiple tissues. The abundance of this protein as well as an abundance of mRNA strongly argues against the expression of such being an epiphenomenon. Instead, we suggest that EAAT5 may have an as yet range of roles in the body, which embrace both the glutamate transport properties of the protein and the co-associated chloride conductance. This latter property, which sets EAAT5 aside from other transporters such as GLAST and GLT-1, may hyperpolarise cells that express such during events that require auto-regulation via modulation of membrane potential, such as regulation of secretion from the goblet cells. These possibilities await further studies.

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