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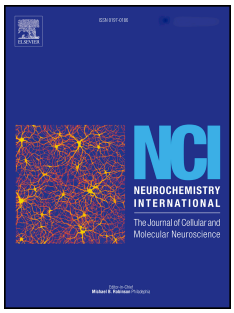

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A novel splice variant of the Excitatory Amino Acid Transporter 5: cloning, immunolocalization and functional characterization of hEAAT5v in human retina.

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ABSTRACT

Excitatory Amino Acid Transporter 5 (EAAT5) is abundantly expressed by retinal photoreceptors and bipolar cells, where it acts as a slow glutamate transporter and a glutamate-gated chloride channel. The chloride conductance is large enough for EAAT5 to serve as an “inhibitory” glutamate receptor. Our recent work in rodents has shown that EAAT5 is differentially spliced and exists in many variant forms. The chief aim of the present study was to examine whether EAAT5 is also alternately spliced in human retina and, if so, what significance this might have for retinal function in health and disease. Retinal tissues from human donor eyes were used in RT-PCR to amplify the entire coding region of EAAT5. Amplicons of differing sizes were sub-cloned and analysis of sequenced data revealed the identification of wild-type human EAAT5 (hEAAT5) and an abundant alternately spliced form, referred to as hEAAT5v, where the open reading frame is expanded by insertion of an additional exon. hEAAT5v encodes a protein of 619 amino acids and when expressed in COS7 cells, the protein functioned as a glutamate transporter. We raised antibodies that selectively recognized the hEAAT5v protein and have performed immunocytochemistry to demonstrate expression in photoreceptors in human retina. We noted that in retinas afflicted by dry aged-related macular degeneration (AMD), there was a loss of hEAAT5v from the lesioned area and from photoreceptors adjacent to the lesion. We conclude that hEAAT5v protein expression may be perturbed in peri-lesional areas of AMD-afflicted retinas that do not otherwise exhibit evidence of damage. The loss of hEAAT5v could, therefore, represent an early pathological change in the development of AMD and might be involved in its aetiology.

KEYWORDS

retina, photoreceptors, glutamate, transporter, EAAT5, alternate splicing.

1 Introduction

In the central nervous system (CNS), L-glutamate (Glu) acts as an excitatory neurotransmitter and as such it is tightly regulated. In the CNS, several glutamate transporters are known to regulate the extracellular levels of Glu; Excitatory Amino Acid Transporter 1 (EAAT1; also called GLAST), EAAT2 (also called GLT-1), EAAT3 (also called EAAC1), EAAT4 and EAAT5. EAAT1 and EAAT2 are mainly expressed in glial cells whereas EAATs 3-5 are located in neurons (reviews: Danbolt, 2001; Sheldon and Robinson, 2007; Lee and Pow, 2010; Zhu and Danbolt, 2013; Šerý et al., 2015). EAAT5 was initially cloned by Arizza et al (1997) and the protein was subsequently shown to be expressed in the mammalian retina, localised mainly to rod photoreceptor terminals and bipolar cells (Pow and Barnett, 2000a). EAAT5 has also been detected in cerebellum (Ochiai et al., 2010) and tissues such as the vestibular system (Dalet et al., 2012) and testis (Lee et al., 2011a). More recent studies of EAAT5 using a newly developed antibody showed abundant expression in many non-nervous tissues including liver, kidney, intestine, heart, lung and skeletal muscle (Lee et al., 2013). Collectively, these findings dispel the widely held notion that the expression of EAAT5 is confined to the retina. We have recently shown that the EAAT5 gene in rodents is subject to alternate splicing and have demonstrated by PCR cloning and sequencing the presence of five novel splice variant forms of EAAT5 which skip either parts of exons or complete exons in the rat retina. Furthermore, we showed that each of these splice variants is expressed at the protein level as assessed by Western blotting using splice-specific antibodies that we have generated (Lee et al., 2012a). However, in this study we re-evaluated these findings with respect to the human retina, since any variant form is of far greater interest to biomedical researchers if it is actually expressed and functional in humans. Surprisingly, we have not found “rodent-type” exon skipping variants of EAAT5 in the human retina but, instead, we identified an abundant novel variant containing an additional exon that has been spliced into the EAAT5 coding region. In this study we have generated a highly specific antibody to this novel splice variant, evaluated its expression pattern in human retina (by immunocytochemistry) in normal and diseased eyes and validated that it is a functional glutamate transporter.

2 Materials and methods

2.1 Sources of human retinae and animal experimentation

Whole eye cups from human cadaveric donors (5 controls, 5 age-matched with dry AMD) were obtained from the Queensland Eye Bank. The eyes were obtained with institutional ethics approval (Metro South Hospital and Health Service Human Research Ethics Committee HREC/07/QPAH/048).

Animal experiments were carried out with ethical permission from the UQ animal ethics committee, in accordance with the NHMRC guidelines for the use of animals in biomedical research and in accord with the ARVO statement for the use of animals in Ophthalmic and vision research.

2.2 Cloning of Human EAAT5 and EAAT5v

Retinal tissues were carefully dissected from post-mortem human eyes and RNA was extracted using TriZol® reagent (Invitrogen, Mount Waverley VIC, Australia) following the manufacturer's instructions. Total RNA (10 µg) from 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. PCR primers were designed to flank the start and stop codons of EAAT5, to amplify the entire coding region of hEAAT5 (GenBank Accession NM_006671.4) and thus identify alternative splicing within the coding region of hEAAT5. PCR was performed using KOD Hot Start DNA Polymerase (Toyobo, Osaka, Japan). The PCR mixture (50µl) contained: 2 µl RT product, 200 µM dNTPs, 0.2 µM 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℃ for 2 min followed by 35 cycles of amplification (95℃ for 20 s, 60℃ for 15 s, 70℃ for 40 s). A blank control (water instead of cDNA template) was used as a negative control in all PCR experiments to confirm that nonspecific PCR amplification was not occurring. Sequences of hEAAT5 sense and antisense primers were F-START: 5'-ATGGTGCTGTGCCCCTTGCC-3' and R-STOP: 5'-GCTCCGCAGGCTCAGACATT-3', respectively; F-START anneals to position 132 of the hEAAT5 cDNA upstream of the START codon and R-STOP anneals to position 1862 corresponding to the STOP condon region which produces a PCR product of 1730 nucleotides. The reaction products were separated on a 0.8 % 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. Plasmids were transformed into *E. Coli* DH5α max competent cells (Invitrogen) and individual colonies were picked and further grown in LB media containing 50 µg/ml Kanamycin for 18-20 hours. Plasmid DNA was extracted from *E Coli* cultures using the Aurum Miniprep kit (Bio-Rad, Hercules, California, USA) and digested with EcoR I restriction enzyme (Genesearch Pty Ltd, Arundel QLD, Australia) to identify plasmids containing inserts. All positive clones (n=20) were sequenced in both directions (by the Australian Genome Research Facility) using the M13 Forward and M13 Reverse primers (Invitrogen).

2.3 RT-PCR screening of hEAAT5v

The novel hEAAT5 splice variant identified in this study was subject to further characterisation by RT-PCR using splice specific primers. PCR (final volume 50 µl) consisted of 1µl of RT reaction mixture, 2 mM dNTP, 0.2 mM sense and antisense primers, 1.5 mM MgCl2, and 2.5U BIOTAQ DNA polymerase (Bioline Pty Ltd, Alexandria, NSW, Australia) in 1X PCR buffer. PCR was performed using the following conditions: initial denaturation at 95ºC for 2 min followed by 35 cycles of amplification (95ºC for 30 s, 62ºC for 30 s and 72ºC for 30s). The reaction products were separated on a 2% agarose gel and visualized by staining with 0.5 µg/ml ethidium bromide (Sigma-Aldrich). Sequences of hEAAT5v sense and antisense primers were F-Insert: 5'-GGTGAGCTTCCCGACAGAGGC-3' and R-STOP: 5'-GCTCCGCAGGCTCAGACATT-3', respectively; F-INSERT anneals to a novel sequence corresponding to an insertion spliced in between exon 7 and exon 8 of hEAAT5v whereas R-STOP anneals to the STOP condon region which produces a PCR product of 735 nucleotides.

2.4 Plasmid construction

Generation of hEAAT5/hEAAT5v constructs was carried out by high-fidelity PCR with primers containing in-frame restriction sites. PCR fragments containing the entire human EAAT5/EAAT5v coding sequences were fused at the EcoR I/Hind III sites in the pHM6 vector (Roche, Castle Hill NSW, Australia) to produce pHM6:hEAAT5 and pHM6:hEAAT5v. Fidelity of the plasmid constructs was confirmed by DNA sequencing.

2.5 Cell Culture and transfections

The COS7 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℃ in 5% CO_2. Cells were plated into 25 cm^2 flasks and when at ~80% confluency, were transfected with DNA constructs [pHM6 empty vector (control) or pHM6-hEAAT5v] using XtremeGene HP (Roche) following the manufacturer's instructions. The transfected cells were cultured for 72-h before being washed gently in sterile PBS (1 min) and then lysed with lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche).

For D-aspartate uptake studies, COS7 cells were plated in 12-well dishes. At ~80% confluency, the cells were transfected with empty pHM6 vector (control) or pHM6:hEAAT5 and pHM6:hEAAT5v using XtremeGene HP (Roche). The transfected COS7 cells were cultured for 3–4 days before performing D-aspartate uptake assays (described below).

2.6 Transport assays

COS7 cells were washed twice with transport buffer containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.5, and 4.5 g/l of glucose. COS7 cells were then incubated in transport buffer containing 20 μM cold D-aspartate plus 2 μCi/ml [^{3}H]D-aspartate (GE Healthcare, Rydamere, NSW, Australia) for 10 min at 25 ℃. Cells were washed three times with ice-cold transport buffer in which NaCl was replaced by choline chloride and solubilized with lysis buffer containing 20 mM Hepes–Tris, pH 7.5, 120 mM NaCl, 1% Triton X-100, 5 mM EDTA. Aliquots of lysed cells were taken for scintillation counting and protein assays. The radioactivity counts were adjusted according to the protein content of the sample and expressed as nmol D-aspartate/mg of protein/min. For affinity constant (K_m) determination, uptake at six different substrate concentrations (5 μM to 100 μM) were performed. All values presented are the mean ± S.D. of experiments performed in triplicate and analysis of kinetic data was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

2.7 Antibodies

Polyclonal EAAT5 antisera against hEAAT5v was generated by immunizing rabbits according to standard protocols (Pow et al., 1993; Pow at al., 2003; Rauen et al., 2004) which we have successfully used to identify splice variants of other EAATs (Lee at al., 2012a, 2012b). The sequence used was KNQVRALQCPRSLPPA and represents a novel

peptide sequence that is generated by the splicing event. Briefly, rabbits were immunized using synthetic peptides coupled to bovine serum albumin (BSA) was conducted using our standard protocols (Pow et al., 2003). Sera were tested by Dot blotting using conjugates of peptides coupled with BSA. The immunizing peptide and controls (synthetic peptides corresponding to other exons in wild-type human EAAT5) were examined. One microliter of conjugate was applied to nitrocellulose membranes (Pall, Cheltenham VIC, 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.

2.8 Immunohistochemistry

Immunoperoxidase labeling for hEAAT5v was performed as previously described using standard methods (Pow and Barnett., 2000a) on vibratome sections of human tissues. Retina tissues were fixed by immersion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 h. Vibratome sections (40 µm thick) of retina were cut, immunolabelled using standard protocols (Pow and Barnett., 2000a), using antiserum at a dilution of 1:5000. Labelling was detected using biotinylated secondary antibodies, streptavidin-horseradish peroxidase complex, and revealed using DAB as a chromogen. Preabsorption of antisera was always used to confirm the specificity of such.

2.9 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, contrast and colour balance adjustments prior to composition of plates using Adobe Freehand.

2.10 Lysate preparation and Western blotting

Retinae were carefully dissected from post mortem human eyes and homogenized in lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche). Protein lysates (~10 µg) were separated on 7% SDS polyacrylamide gels 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 2-3 h and then incubated in fresh blocking buffer containing primary antibodies overnight at 4º C. After 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 EAAT5v antiserum. The peptide was added to the diluted antibody and incubated at 4^0C with gentle shaking for 9 hours, prior to use.

2.11 Deglycosylation

Human retinal lysate (50 µg) was re-suspended in reaction buffer (pH 7.5), containing 20 mM NaH_2PO_4, and 1% NP-40. N-Glycosidase F (0.1U; from *Flavobacterium meningosepticum*; Sigma-Aldrich) was added and the suspension was incubated for 18 hr at 37℃. The samples were analyzed by Western blot analysis as described above. Controls were processed as above but without the addition of N-glycosidase-F.

2.12 Analysis of potential transmembrane topology of hEAAT5v

Data derived from sequencing of hEAAT5v was subject to analysis using the online TMPRED algorithm, which predicts potential transmembrane topologies of proteins; http://www.ch.embnet.org/software/TMPRED_form.html.

2.13 Quantification

Image analysis of DNA gels was performed using NIH Image J.

3 Results

3.1 Cloning of a novel splice variant of EAAT5 in human retina

RT-PCR performed on retinal mRNA extracted from human donor eyes produced two abundant PCR products, a ~1.7 Kb fragment corresponding to wild-type hEAAT5 and a slightly larger ~1.9 Kb fragment corresponding to a hEAAT5 splice variant. Relative abundance of this novel splice variant is ~30% of wild-type hEAAT5 (Fig 1A). Cloning and sequence analysis of the PCR products indicated that they corresponded to two distinct mRNAs: the ~1.7 Kb fragment encoded the expected original hEAAT5 mRNA (NM_006671.4) whilst the ~1.9 Kb fragment encoded a hEAAT5 mRNA not described previously, which is referred to herein as hEAAT5v. This novel transcript contained an additional 177 nucleotide (in-frame) insertion spliced in between exon 7 and exon 8. The sequence of this novel 177 nucleotide insertion corresponded to part of the intron 7 sequence of hEAAT5 gene. The squence for hEAAT5v was deposited in GenBank (JF917096). The expression of hEAAT5v transcript was further confirmed by RT-PCR using sequence-specific primers that selectively amplify the novel insert (Figure 1B). Fig. 2A depicts the exon organization of hEAAT5 and hEAAT5v mRNAs.

3.2 Prediction of transmembrane topologies

Figure 2B graphically represents the predicted transmembrane topologies for wild-type hEAAT5 and hEAAT5v. Wild type hEAAT5 is predicted as having eight transmembrane domains (TMD) and two re-entrant loops of opposite orientation with a molecular weight of ~63 kDa. hEAAT5v is predicted using the same algorithm as having ten TMD with a predicted mass of ~67 kDa; the additional 59 amino acids (predicted to be extracellular) inserted after exon 7 is predicted to expand the re-entrant loop, resulting in the formation of an extracellular domain.

3.3 Functional properties of hEAAT5v

D-aspartate uptake studies were performed to determine if cells transfected with hEAAT5 or hEAAT5v were able to transport this non-metabolizable Glu analogue. Expression of hEAAT5v in COS7 cells resulted in a significant increase in D-aspartate uptake over mock transfected cells and this was also observed for wild-type hEAAT5 (positive control) (Fig 3A). Uptake by hEAAT5 and hEAAT5v was inhibited by TBOA which competitively blocks all EAAT family transporters (Shimamoto et al. 1998). The modest extent of the observed

increases in D-aspartate uptake for both hEAAT5 and hEAAT5v are consistent with data from recent studies showing that EAAT5 is a slow, low capacity glutamate transporter (Gameiro et al., 2011; Schneider et al 2014). The apparent K_m value for D-aspartate was ~54 μM (Fig 3B), which is in agreement with the previously reported value for hEAAT5 (Arriza et al., 1997). Therefore, hEAAT5 and hEAAT5v both have similar substrate affinities.

3.4 Specificity of the hEAAT5v antibody

Specificity of the hEAAT5v antibody for its target was tested on Dot blots of the initial immunizing peptide; the antiserum specifically detected the target peptide but not irrelevant peptides (Data not shown). To further assess antibody specificity, COS7 cells were transfected with an empty plasmid (control) or plasmids encoding hEAAT5 or hEAAT5v. Western blotting was performed on lysates from the transfected cells to detect specificity of antibody-antigen interaction. The hEAAT5v antibody detected a single band migrating at ~67 kDa in lysates of cells transfected with hEAAT5v but not in lysates of cells transfected with hEAAT5 or the empty plasmid (Fig 4A). The relative mass of the immunoreactive band corresponded to the predicted molecular weight of hEAAT5v (67,020 Da; predicted by ExPASY Bioinformatic Resource Portal). An antibody directed against the C-terminal region of hEAAT5 produced bands of ~62 kDa and ~67 kDa in lysates of cells transfected with hEAAT5 and hEAAT5v, respectively (Fig 4B). These results demonstrate that the hEAAT5v antibody does not recognise hEAAT5 since the presumed loop of hEAAT5v used as the antigen is completely novel. To investigate whether glycosylation was involved in post-translational processing of hEAAT5v, human retinal extract was treated with N-glycosidase F, which removes all N-glycans. Western blot analysis (Fig. 4C) demonstrated that digestion with N-glycosidase F had no apparent effect on the Molecular weight of hEAAT5v, however, in positive control experiments, treatment of retina extracts with N-glycosidase F reduced the size of EAAT1 (data not shown) confirming that the experimental conditions were appropriate. These findings suggest that hEAAT5v is not N-glycosylated.

3.5 Immunolocalization of hEAAT5v in human retina

Immunocytochemistry of sections of human retina revealed strong immunoperoxidase labelling for hEAAT5v in all photoreceptors (Fig 5A). Labelling was associated with the inner segments, cell bodies and synaptic terminals (Fig 5B) of the photoreceptors, with

particularly intense staining of rod photoreceptor terminals. Observations on retinas from eyes afflicted with AMD revealed a change in distribution, with the normal areas of the eye retaining immunostaining for hEAAT5v, but in the perilesional area there was a gradual diminution of immunostaining, with no immunostaining in the lesioned area (Figs 5C, 5D, 5E). These data indicate that obviously in areas where there are no, or few photoreceptors, hEAAT5v is absent, but there is also loss of immunoreactivity from the photoreceptors in the peri-lesion region that have not died but are at imminent risk of death if the lesion were to expand.

4 Discussion

4.1 hEAAT5v – a new form of EAAT5

In this study we demonstrate a new and relatively abundant form of EAAT5 in the human retina, which we refer to as hEAAT5v. Unlike any of the other alternately spliced forms of EAAT5 that are found in rodents, which lose parts of the EAAT5 protein, this human form has, instead, an additional insert within a predicted extracellular loop. This newly identified form of EAAT5 is functional as a glutamate-transporting protein as evinced by its capacity to transport D-aspartate, which was comparable to the transport capacity of normal hEAAT5. hEAAT5v is demonstrably expressed in the retina at the protein level as assessed by Western blotting and immunocytochemistry. The predicted mass of hEAAT5v is ~67kDa. The protein detected with our new antibody runs at this size in Western blots, supporting the view that we are detecting this protein. Similarly, cells transfected with plasmids containing the hEAAT5v insert yield a protein that also runs at this size in Western blots. Many EAATs such as EAAT1 are glycosylated (review: Danbolt 2001). Using the NetNGlyc 1.0 glycosylation prediction tool there is a predicted N-glycosylation site at residue 254; this residue is, however predicted to be intracellular rather than extracellular in our proposed membrane topology model and thus should not be glycosylated. This accords with the experimental data where we were unable to demonstrate any size shift after deglycosylation of the protein sample. We therefore conclude that hEAAT5v is not N-glycosylated.

The model we propose for the membrane topology is based upon known data for closely related members of this transporter family (Jiang and Amara 2011), particularly EAAT2, where the proteins have 8 transmembrane domains, and potentially two re-entrant loops. The models predict that the exon 7 insert creates a new extracellular loop as an expansion

of one of the re-entrant loops. The data from our D-aspartate uptake experiments indicate that the capacity of hEAAT5v to transport D-aspartate is unaffected by this new proposed loop structure. Furthermore, the calculated K_m value for hEAAT5v is similar to that of hEAAT5, suggesting that this loop structure does not alter substrate affinity. This accords with prior data (Ryan and Vandenberg 2005) showing that the critical molecular determinants for glutamate binding and translocation are associated with residues coded by exon 9 (encompassing the second re-entrant loop) and similarly the key molecular determinants for chloride permeation are associated with transmembrane region 2. These data support the observation in this study that hEAAT5v is a functional variant of EAAT5 with functional glutamate transporting properties, and that it should, in the absence of secondary impact, also exhibit chloride-conducting properties.

Other members of the mammalian EAAT family have also been shown to have potential inserts (Meyer et al 1999) but, in general, these have been predicted to yield frame shifts and thus cause truncations; accordingly they have not been fully characterised or anatomically localised, and so there is a paucity of supporting data for the nature of the impact of this type of insert on function of the transporter. This study appears to be the first instance where a mammalian EAAT with an insert has been shown to be functional, expressed at the protein level and localised to a population of neurons which are known to use EAAT5 as one of their normally expressed EAATs.

4.2 Relevance to human disease

We have previously shown that there is perturbation of many EAATs in a variety of disease states such as AMD. Thus, expression of the main Müller cell glutamate transporter GLAST (EAAT1) is reduced in and around AMD lesion sites (Pow et al 2005). As GLAST is responsible for much of the overall glutamate homeostasis in the retina (Pow et al 2000b) it is reasonable to assume that there is glutamate dysregulation in and around sites of AMD lesions. Similarly, we have previously shown (Pow et al., 2005) that there is a perturbation of GLT1 expression in photoreceptors in the perilesional areas of AMD retina. Whether the same underpinning mechanism is responsible for down-regulating both GLAST in Muller cells and GLT1 and EAAT5 in photoreceptors is open to conjecture, but loss of GLAST, GLT1 or EAAT5 would yield the same outcome, i.e. signalling with glutamate by the photoreceptors around lesions would be highly impaired. The loss of hEAAT5v in the peri-lesion areas of the AMD retina is significant because these are the cells that are at risk as the AMD lesion expands. Accordingly, we suggest that hEAAT5v

could be a component of early pathological changes in AMD and thus by inference, its targeting could produce novel therapies for AMD. It will be interesting in future immunocytochemical studies to determine whether expression of the “normal” form of EAAT5 is also perturbed in peri-lesional areas of AMD-afflicted retinas or if there are additional pathology-related changes to EAAT5 splicing.

4.3 EAAT5 splicing in the human retina is different to that observed in the rodent retina

When this study was initiated, we expected to detect exon-skipping variants of EAAT5 such as those we have previously detected in the rodent retina (Lee et al 2012a). However, the human retina does not appear to express such exon-skipping forms. Instead, a single dominant variant form, namely hEAAT5v was detected. Our search of the NCBI database did reveal two referenced sequences that were derived from a human retinoblastomas cell line. These include a variant (called transcript variant 5; NR_109858.1) which is referenced as non-coding since the use of a 5’-most expected AUG and a 28 nucleotide deletion from an internal exon would render it a candidate for nonsense mediated mRNA decay. Another is a variant (called transcript variant 3; NM_001287597.1) that lacks exon 3 but contains a 19 nucleotide insertion following exon 10 which is predicted to encode a smaller isoform. To the best of our knowledge, we have not detected such exon-skip variants in any of our PCR screening using human retinas (from normal controls or those afflicted with AMD) despite having compatible hEAAT5 forward and reverse primers; we have also performed PCR amplification using higher cycle numbers (36 to 38 cycles) and have not detected such variants apart from the bands corresponding to hEAAT5 and hEAAT5v. The reason for this disparity may be attributed to the cell type used (i.e. retinoblastoma cell line vs human retinal tissue) and further suggests that such transcripts may be the result of aberrant splicing of the EAAT5 gene in neuroblastoma.

From this we infer that the control of splicing in the human retina is somewhat different to that in the rodent. This distinction strongly supports the view that whilst rodent models are critical for most biomedical research, it is imperative that actual human tissues are evaluated to confirm the significance and relevance of rodent data. This is particularly important in situations such as this study where a functional protein is generated in the human, but the exon skip forms observed in rodents may not be active transport proteins.

Our observation that bipolar cells do not express hEAAT5v indicates that by inference, bipolar cells probably express the “normal” form of EAAT5, namely hEAAT5. Unfortunately, antibodies that we (and others) have previously made to the “normal” form of EAAT5 do not discriminate between hEAAT5 and hEAAT5v, so we cannot unequivocally conclude if hEAAT5 is the bipolar cell form of EAAT5 and hEAAT5v is the sole photoreceptor form of EAAT5. This is however a plausible hypothesis as we have previously demonstrated that for EAAT2 (GLT1), each of the key alternately spliced forms is expressed in a distinct cell type i.e. GLT1a is in amacrine cells, GLT1b is in some bipolar cells and cone photoreceptors (Reye et al., 2002) whilst GLT1c is in photoreceptor terminals (Rauen et al 2004). The resolution of this question as to whether there is complete or partial segregation of differently spliced forms of EAAT5 in different cells awaits the development of new antibodies.

5 Conclusions

We have identified a novel functional splice variant of EAAT5 in the human retina, referred to as hEAAT5v where the open reading frame is expanded by insertion of an additional exon. Immunocytochemistry demonstrated labelling of this novel protein in photoreceptors in human retina. In retinas afflicted with AMD, there was a loss of hEAAT5v from the lesioned area and from photoreceptors adjacent to the lesion; this may represent an early pathological change in the development of AMD and might be involved in its aetiology.

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Figure legends

Figure 1

Identification of a novel variant of hEAAT5 in human retina. (A) RNA from human retina extracted from donors 44 and 88 years of age (RET1 and RET2, respectively) were used for RT-PCR with hEAAT5 specific primers. Analysis of PCR products on a 0.8% agarose gel showed ~1.7 Kb and ~1.9 Kb fragments corresponding to wild-type hEAAT5 and a novel variant (hEAAT5v) were detectable in retina. Relative abundance of hEAAT5v transcript (compared to wild-type hEAAT5) is shown in the bar graph. (B) mRNA encoding hEAAT5v is detectable in human retina by RT-PCR using hEAAT5v specific primers.

Figure 2

mRNA and secondary structure of hEAAT5v. (A) Schematic diagram showing exon structure of wild-type hEAAT5 mRNA and hEAAT5v mRNA. Exons are depicted by rectangles; UTR = untranslated region; AUG = START codon; UGA = STOP codon; hEAAT5v is a longer variant containing a 177 nucleotide insertion (7-int) between exon 7 and exon 8. (B) Schematic diagram showing predicted transmembrane topologies of wild type hEAAT5 and hEAAT5v; the extracellular surface and the plasma membrane are indicated. Exon boundaries are overlayed onto the predicted topologies to demonstrate the predicted changes in topology that result from insertion of the additional 59 residues present in hEAAT5v.

Figure 3

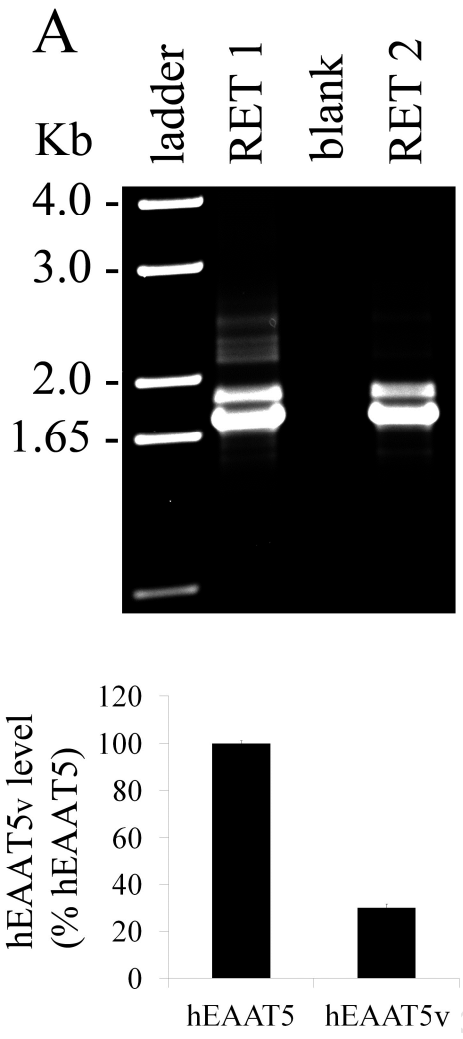
Uptake of [^{3}H] D-aspartate into COS7 cells. (A) Controls were COS7 cells transfected with empty plasmid; enhanced uptake was evident in cells transfected with full length hEAAT5 and hEAAT5v plasmids which were blocked by 100 μM TBOA. (B) Kinetic analysis of hEAAT5v-mediated D-aspartate uptake in COS7 cells. Kinetic characteristics were determined at substrate concentrations ranging from 5 to 100 μM. The data represent uptake into hEAAT5v transfected cells minus uptake into empty vector-transfected cells. Values (mean ± S.D. n = 3) were fitted to a curve by nonlinear regression analysis using Graph Pad Prism 7 and generated values of 54.32±15.02 for the K_m.

Figure 4

Specificity of the hEAAT5v antibody. (A) COS7 cells were transfected with an empty plasmid (control) or plasmid encoding hEAAT5 or hEAAT5v. Cell extracts were resolved by SDS-PAGE and immunoblotted with the hEAAT5v antibody; the hEAAT5v antibody detected a band of ~67 kDa in hEAAT5v transfected cells but not in hEAAT5 transfected cells or control cells. (B) Western blot of the same samples with an antibody directed against the C-terminus of EAAT5 which recognises both isoforms shows change in molecular weight. (C) Western blot showing the detection of hEAAT5v in the human retina; Deglycosylation with N-glycosidase F did not result in any size change for hEAAT5v.

Figure 5

Immunolabelling for hEAAT5v in human retina. In panel A, strong labelling is evident in photoreceptor terminals in the outer plexiform layer (OPL). Labelling is also associated with photoreceptor somata in the outer nuclear layer (ONL) and the inner segments of photoreceptors. Panel B shows immunostained rod photoreceptor terminals (black arrow) whilst red arrow indicates a structure (delineated by a red line) which we interpret as being a more weekly stained cone terminal. Panel C illustrates section of retina from an eye with AMD. In the “normal region” photoreceptors display hEAAT5v labelling, with progressive loss of labelling in the “peri lesions area” (see Panel D) despite no obvious loss of photoreceptors in this region whilst in the lesioned area there is extensive photoreceptor loss and consequently loss of immunostaining, the darker “stained" cells (panel E) representing macrophages and other infiltrating cells which express endogenous peroxides. Scale bars, A, 50 µm, B, 5µm, C, 200 µm, D, 100 µm, E, 50 µm.



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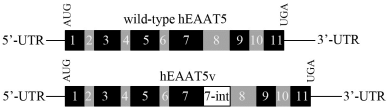

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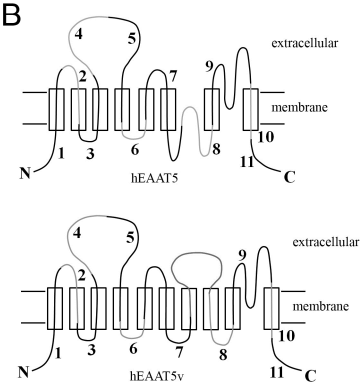

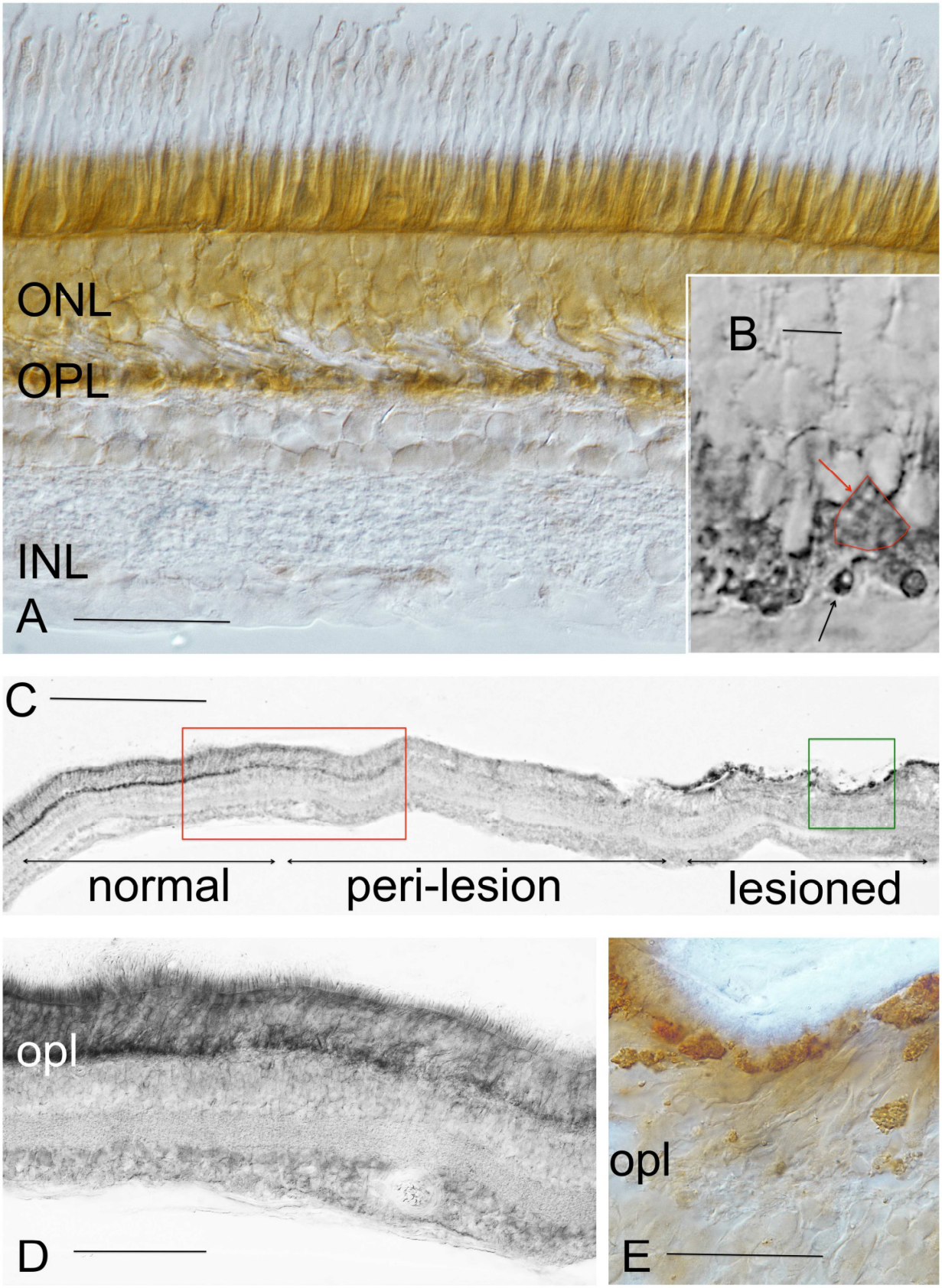
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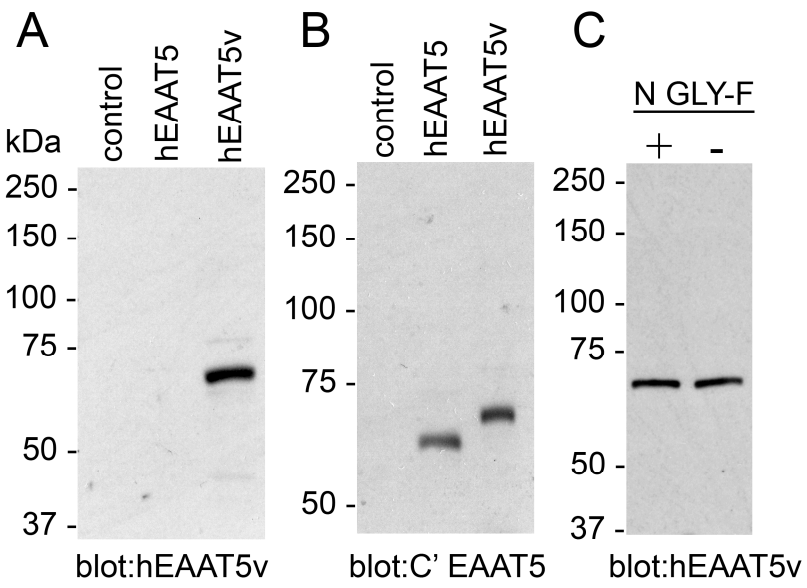

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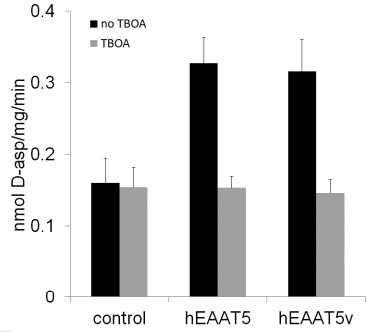

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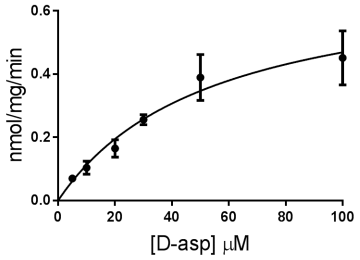

Highlights

- We have identified a novel splice variant of EAAT5 in human retina, referred to as hEAAT5v which contain an additional exon.
- hEAAT5v is a functional glutamate transporter and abundantly expressed in photoreceptors.
- In retinas afflicted with age-related macular degeneration (AMD), hEAAT5v is lost from the lesioned area and from photoreceptors adjacent to the lesion; this may represent an early pathological change in the development of AMD and might be involved in its aetiology.