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Regional differences in glutathione accumulation pathways in the rat cornea: mapping of amino acid transporters involved in glutathione synthesis

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Abstract

In this study we have sought to complete the identification and localization of uptake pathways involved in accumulating precursor amino acids involved in GSH synthesis in the rat cornea. To do this, we performed reverse transcription PCR (RT-PCR) to identify glutamate transporters (EAAT 1-5) and glycine transporters (GLYT 1-2) at the transcript level. Western blotting was used to verify protein expression, while immunolabelling of sagittal sections was used to localise transporters to the different layers of the cornea. Immunolabelling of en face sections was used to examine the subcellular distribution of proteins in the corneal endothelium. Our findings revealed EAAT 1-5 and GLYT 1-2 to be expressed at the transcript and protein level in the rat cornea. Immunohistochemistry revealed all amino acid transporters to be localized to the epithelium. In the majority of cases, labelling was restricted to the epithelium, and labelling absent from the stroma or endothelium. However, EAAT 4 and GLYT 2 labelling was detected in the stroma with EAAT 4 labelling also present in the endothelium. Overall, the identification of amino acid transporters strongly supports the existence of an intracellular GSH synthesis pathway in the rat corneal epithelium. This suggests that regional differences in GSH accumulation pathways exist, with direct uptake of GSH and intracellular synthesis of GSH restricted to the endothelial and epithelial cell layers, respectively. This information is important in the design of targeted strategies to enhance GSH levels in specific layers of the cornea to prevent against oxidative damage, corneal swelling and loss of corneal transparency.
1. Introduction

Since the cornea is positioned at the very front of the anterior eye, it requires a robust antioxidant defence system to minimise oxidative stress caused by ultraviolet (UV) radiation (sunlight), pathogens and other environmental toxins. There are a number of antioxidant enzymes that are utilised to detoxify ROS that include superoxide dismutase, catalase and glutathione peroxidase (Chen et al., 2009). In addition, two important non-enzymatic antioxidants exist in the cornea; ascorbic acid and glutathione (GSH). Ascorbic acid (Vitamin C) is found in high concentrations in the corneal epithelium (Brubaker et al., 2000), where it acts to absorb UV radiation (Ringvold, 1996). While ascorbic acid is found at higher levels in the epithelium compared to GSH (Brubaker et al., 2000; Riley and Yates, 1977), GSH is essential for reducing oxidised ascorbic acid to ascorbic acid to maintain ascorbic acid levels (Wu et al., 1998). GSH and oxidised glutathione (GSSG) are also essential for protecting cellular integrity and pump function in the corneal endothelium and thus regulating stromal deturgescence (Anderson et al., 1974; Araie et al., 1988; Nakamura et al., 1994; Riley, 1984). At low or physiological concentrations of $H_2O_2$ in the aqueous humour, it has been shown that GSH is preferentially used to protect and maintain endothelial integrity (Umapathy et al., 2013), further highlighting the importance of GSH in the cornea.

GSH is a tripeptide synthesised from cysteine, glutamate and glycine by the sequential actions of the enzymes $\gamma$-glutamylcysteine synthetase ($\gamma$-GCS) and glutathione synthetase (Rathburn, 1980; Valencia et al., 2001). Cysteine is the rate limiting substrate for GSH synthesis (Davis et al., 1993; Deneke and Fanburg, 1989) and $\gamma$-GCS the rate limiting enzyme for GSH synthesis (Richman and Meister, 1975). GSH levels are maintained via a combination of GSH uptake, de
*novo* synthesis from its precursor amino acids, GSH regeneration from GSSG by glutathione reductase and GSH efflux (Lash, 2009). However, while it is well established that GSH plays an important role in maintaining normal cornea function, less is known about the identity of molecular pathways utilised to accumulate GSH in the cornea.

To address this, we have previously mapped markers of GSH uptake, synthesis and efflux pathways in the different layers of the rat cornea (Li et al., 2012). We identified regions of the cornea involved in GSH synthesis by mapping the localisation patterns of GSH, cysteine, γ-GCS, and the cystine/glutamate antiporter (System Xc-), regions involved in GSH uptake by localising putative GSH uptake transporters; the organic anion transporter (OAT1 and OAT3) and the sodium dicarboxylate 3 transporter (NaDC3), and regions involved in mediating GSH efflux by mapping the expression of members of the multidrug resistance associated proteins (MRP1, 2, 4 and 5)(Li et al., 2012). From these studies, our results show that regional differences of GSH uptake, synthesis and efflux pathways exist in the epithelium and endothelial layers. The strong localization of γ-GCS, cysteine and System Xc-, all key components of GSH synthesis, to the epithelium, but not the endothelium, suggests that GSH synthesis is the primary source of GSH in the corneal epithelium (Li et al., 2012). In contrast, the endothelium appears to rely on GSH accumulation from the aqueous humour, since it expresses transporters implicated in the uptake of GSH (Li et al., 2012). While the epithelium lacks these GSH uptake mechanisms, it does express MRP4 and 5 (Li et al., 2012) which in other ocular issues are able to mediate efflux of molecules conjugated to GSH to detoxify reactive endogenous metabolites or remove xenobiotics (Pelas and Delamere, 2009; Sharma et al., 2003). Overall, our approach of mapping key components of the different GSH accumulation pathways is a strong indication that regional
differences in GSH metabolism exist in the cornea which are consistent with the different contributions these cell layers play to overall corneal function.

In this study, we aimed to extend and complete our previous work to identify and localise the remaining uptake pathways involved in accumulating glutamate and glycine for GSH synthesis. To achieve this, reverse transcription PCR, western blotting and immunohistochemistry were employed to determine whether specific transporters exist at the transcript or protein level, and then immunohistochemistry was utilized to localise candidate transporters to the different layers of the cornea. Specifically, we have focused on identifying the glutamate transporters (EAAT 1-5), and the glycine transporters (GLYT 1, GLYT 2) to complete our molecular inventory of GSH accumulation pathways in the rat cornea. This completed inventory provides insights into the endogenous pathways utilised by the cornea to accumulate GSH and thus protect itself from oxidative stress.

2. Materials and Methods

2.1 Reagents

Phosphate-buffered saline (PBS) was prepared from PBS tablets (Sigma-Aldrich, St Louis, Missouri, USA). Antibodies for the Excitatory Amino Acid Transporters 1-5 (EAAT1-5) and the Glycine transporters 1-2 (GLYT1-2), and their corresponding control peptides were all purchased from Alpha Diagnostic International (San Antonio, Texas, USA). The β-actin antibody was purchased from Abcam (Cambridge, UK). Horse radish peroxidase (HRP)-conjugated secondary antibody was purchased from Amersham, GE Healthcare (Waukesha, Wisconsin, USA). Alexa Fluor® 488 and 568 goat anti-rabbit antibodies were purchased from Life Technologies.
(Carlsbad, California, USA), phalloidin Alexa Fluor®488 from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and propidium iodide (PI) from Sigma-Aldrich (St Louis, Missouri, USA). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich.

2.2 Animals

All animals were treated according to the ARVO Statement for the use of Ophthalmic and Vision Research using protocols approved by the University of Auckland Animal Ethics Committee. Twenty-one day old Wistar rats were sacrificed by CO₂ asphyxiation. The eyes were removed and corneas dissected for reverse transcriptase PCR or western blotting, or whole eyes processed for immunohistochemical experiments.

2.3 Reverse Transcription-Polymerase Chain Reaction

Forty-six rat corneas were dissected and pooled together, along with one brain and one heart (positive control tissues) and placed in RNAlater™ tissue storage reagent (Invitrogen™, Carlsbad, California, USA) before total RNA isolation. Total RNA was isolated with Trizol® (Gibco, New York, USA) according to the manufacturer’s protocols. Genomic DNA was removed from the total RNA before cDNA synthesis by a 20 minute incubation at 20°C with 0.1 U/µl DNase I (Roche Molecular Biochemicals, Basel, Switzerland). mRNA was purified using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotechnology, Piscataway, New Jersey, USA). First strand synthesis and cDNA amplification were performed with a ThermoScript™ RT-PCR system (Invitrogen™, Carlsbad, California, USA). cDNA was synthesised from 1 µg RNA with 5 µM oligo(dT)₂₀ in 10 µl final reaction volumes. The RNA was denatured at 65°C for 5 minutes and then placed on ice to cool before adding 10 µl of the following mix to give final
concentrations of 1 x cDNA synthesis buffer, 0.5 mM dithiothreitol (DTT), 1 mM dNTPs (dATP, dTTP, dCTP and dGTP) and 15 U/µl reverse transcriptase (Invitrogen™, Carlsbad, California, USA). A control reaction (no cDNA synthesis) was also conducted in the absence of reverse transcriptase. Synthesised cDNA (1 µl) or control reaction (1 µl) were added to separate PCR mixtures. The reaction mixture contained 1 x PCR buffer, 1 mM MgCl₂, 0.2 mM dNTPs, 0.05 U/µl Platinum® Taq DNA polymerase and 0.2 µM sense and anti-sense primers from the primer sets listed in Table 1. The DNA polymerase was heat activated at 94°C for 10 minutes prior to PCR cycling. Amplification was performed for 35 cycles with each cycle consisting of a 30 second denaturation at 94°C, a 45 second annealing step at the indicated temperature (see Table 1), followed by a 45 second extension at 72°C. After the final cycle, a 10 minute extra extension period at 72°C was performed to optimise ligation conditions. Amplified PCR products were analysed by electrophoresis on 0.8% agarose gels and subsequently sequenced. The primer sets and the expected sizes of PCR products are listed in Table 1.

2.4 Protein preparation and protein concentration determination

Sixteen corneas were extracted and pooled together from twenty-one day old Wistar rats, along with one brain and one heart (positive control tissues) and homogenised using an electric drill followed by Terumo hypodermic needles of 20, 23, and 25 gauge in succession in homogenising solution (5mM Tris-HCl (pH 8.0), 5mM EDTA and 5mM EGTA containing protease inhibitors). Tissue homogenates were then centrifuged at 12,000 rpm for 20 minutes at 4°C. The pellets were washed three times in storage solution with centrifugation. The resultant crude membrane protein fractions were re-suspended in storage solution (500µL for heart tissue, 500 µL for cerebellum and 200µL for corneal tissue) and stored at -80°C. Protein concentration was determined using a
Direct Detect® spectrometer fitted with a Fourier transform infrared (FTIR) system (Merck Millipore, Darmstadt, Germany).

### 2.5 Western blotting

15µg protein was incubated in loading dye containing β-mercaptoethanol and separated on a 10% SDS-PAGE gel. Protein bands were transferred to a PVDF membrane by electrophoresis for 40 mins at 170mA and then incubated with blocking solution (5% milk in Tris-buffered saline containing 0.1% v/v Tween® 20 (TBS-T; 2mM Tris–HCl, 140mM NaCl, pH 7.6 plus Tween® 20) at room temperature for 1 hour. The protein blots were incubated overnight with either EAAT 1-5 isoform specific antibodies (1:200) or GLYT1-2 isoform specific antibodies (1:1000) diluted in antibody solution (2mM EDTA, 150mM bovine serum albumin (BSA), 1 x TBS-T) for 1 hour. Peptide control experiments for all transporters followed identical procedures, except that the primary antibodies were incubated with at least 10 to 20-fold excess of their antigenic peptide for 2 hours at 37˚C, followed by overnight incubation at 4˚C. The complexes were then pelleted by centrifuging at 12,000 rpm for 15 minutes and the supernatant added to the PVDF membranes. Following washes in TBS-T, PVDF membranes were incubated with anti-rabbit HRP conjugated secondary antibody for 1 hour each. After incubation, the membrane was washed three times for 10 minutes in TBS-T. Labelled protein was visualised by chemiluminescence detection (ECL Select; Amersham) using a FujiFilm LAS-4000 Scanner (Fujifilm Life Science, CT). Equal protein loading was tested by stripping the PVDF membranes with 2% SDS, 100mM β-mercaptoethanol, 62.5mM Tris (pH 6.7) to remove the antibodies but retain the transferred proteins and re-probing the membrane with antibodies to detect β-actin (1:10,000).
2.6 Immunohistochemistry

To image all three layers of the cornea (epithelium, stroma and endothelium), whole eyes were oriented in an equatorial plane and cryosectioned to obtain 13-15µm thick sections. Sections were collected onto Superfrost™ Plus microscope slides (Thermo Fisher Scientific) and then fixed for 1 hour at room temperature in 0.75% paraformaldehyde (PFA) for labelling with antibodies. Sections were washed three times and then incubated in blocking solution (3% BSA and 3% normal goat serum) for 1 hour to reduce non-specific labelling. The sections were then labelled with either EAAT 1-5 (1:100) or GLYT 1-2 (1:5000), antibodies diluted in blocking solution overnight at 4°C. Sections were washed and then incubated in secondary goat anti-rabbit Alexa 488 (1:200) for 2 hours each. Control sections omitting the primary antibodies, or by incubation of primary antibodies with their corresponding antigenic peptides were also prepared. Cornea morphology was visualized with the cell nuclei marker, propidium iodide (100µmol/l) in PBS, for 1 hour. Sections were washed and mounted with VECTASHIELD® HardSet™ fluorescent mountant (Vector Laboratories, Burlingame, California, USA), and viewed using a confocal laser scanning microscope (Olympus FV100, Tokyo, Japan) with FluoView 2.0c software. Images were pseudo-coloured using Adobe Photoshop software.

To image the corneal endothelium, en face preparations of the cornea was performed. A whole rat eye was enucleated and a continuous cut made around the circumference of the corneoscleral junction to trim the scleral and retinal layers and expose the posterior surface of the lens. The anterior eye cup was fixed in 4% PFA for 1 hour. The hardened lens was then extracted from the eye cup and the cornea dissected out. The cornea was then fixed for a further hour before being washed three times in PBS, for five minutes each. The cornea was permeabilised with 1% w/v
Triton X-100 in PBS for 20 minutes at room temperature. The cornea was then washed in PBS three times, for five minutes each, before being incubated in the selected primary antibody overnight at 4°C, followed by secondary antibody for two hours in the dark at room temperature. The cornea was washed repeatedly in PBS before being incubated with Phalloidin Alexa Fluor®488, in PBS, for an hour in the dark at room temperature, to highlight the F-actin filaments of the corneal endothelial cells. After an additional washing in PBS, the cornea was placed endothelial side up and flattened under a binocular dissecting stereo microscope (Leica EZ4 Microsystems) by making five radial incisions along the periphery with a sharp sterile blade. The whole cornea preparation was then mounted with VECTASHIELD® HardSet™ fluorescent mountant (Vector Laboratories) and sections viewed by confocal microscopy.

3. Results

3.1 Identification and localization of GSH precursor amino acid transporters

(i) Glutamate uptake transporters: EAAT 1-5.

EAATs are known to uptake glutamate and it has been proposed that EAATs work in combination with System Xc- to maintain the glutamate concentration gradient required to drive cystine uptake (Li et al., 2012). However, it is also possible that EAATs could be used to accumulate glutamate directly for GSH synthesis. In order to determine which of the EAAT isoforms were present in the rat cornea, RT-PCR was performed on mRNA extracted from whole corneas using isoform specific primers for EAAT 1, 2, 3, 4 or 5. PCR products of an appropriate
size were amplified for all EAAT isoforms from cornea mRNA as well as from mRNA extracted from control tissue verifying primer specificity (Figure 1A). PCR products were sequenced and found to correspond to their respective GenBank sequences, thereby establishing EAAT 1-5 to be present at the transcript level in the rat cornea.

We next investigated whether all five EAAT isoforms were present at the protein level by western blotting. Each western blot represents bands from positive control tissue (brain or heart) and cornea tissue following probing with an EAAT isoform specific antibody (Ab). Bands of the reported molecular weight for a specific candidate transporter are highlighted with an arrowhead and additional bands highlighted with an asterisk (*). To determine the specificity of a band, an additional western blot was probed with the primary antibody of interest preabsorbed with an excess of corresponding control peptide. If a band is specific for the transporter protein of interest, we expect this band to be absent in the antibody plus control peptide blot (Ab + CP). Finally, below each western blot, we have also included a blot for β-actin to determine equal loading of protein between lanes.

Our findings show that all five EAAT isoforms are expressed at the protein level in the rat cornea (Figure 1B-F). A common feature of our western blots was the difference in masses for EAAT isoforms between brain/heart versus cornea tissue. In Table 2, we have summarised the predicted versus observed molecular masses of the EAAT isoforms and searched the literature for what the observed bands, if different from the predicted mass, may represent. In the brain or heart, the observed bands detected typically result from post translation modifications of the EAAT protein such as glycosylation or truncation. In the cornea, the predicted band masses
tended to correspond with the observed band sizes, suggesting that the EAAT protein undergoes less post translational processing in the cornea compared to that of the brain or heart.

Having established that EAAT 1-5 are expressed at the protein level in the rat cornea, we next investigated their localisation in the different layers of the cornea (epithelium, stroma and/or endothelium). To do this, we double labelled sagittal corneal sections with the nuclear marker propidium iodide (red) and an EAAT isoform specific antibody (green) (Figure 2). We imaged the peripheral and central cornea to ensure that the labelling patterns we observed were maintained throughout the entire length of the cornea. However, for all of the EAAT isoforms, (and the GLYT transporters), labelling in the two different regions were identical and so we have included representative images obtained from the central cornea only. We also performed control peptide experiments in parallel to confirm specificity of labelling for a specific EAAT isoform (Figure 2, insets). In all cases, labelling was knocked down, indicating staining to be specific for each EAAT isoform.

Representative overview images for each EAAT isoform revealed EAAT 1, EAAT 2, EAAT 3 and EAAT 5 to be localised specifically to the epithelium, with no labelling detected in the stroma or endothelium (Figure 2B-D & F). On the other hand, EAAT 4 was revealed to label the epithelium, stroma and endothelium (Figure 2E). In order to more closely assess the localisation of EAATs within a specific region of the cornea, high magnification images were captured of the endothelium, stroma and epithelium and the results presented below:

Endothelium
Of all the EAAT isoforms, EAAT 4 was the only one detected in the endothelium (Figure 3A). To get a better idea of the subcellular localisation of EAAT 4 within this region, we labelled flat en face sections with phalloidin (red) to highlight the actin cytoskeleton and EAAT 4 antibodies (green) (Figure 3B). It can be seen that labelling of EAAT 4 was cytoplasmic (Figure 3B), and not associated with the membrane.

**Stroma**

Of all the EAAT isoforms, EAAT 4 was also the only isoform to label the stroma (Figure 4A). A closer examination of this region reveals EAAT 4 to possess a distinctive labelling pattern (Figure 4B), reminiscent of the spindle shaped, long processes possessed by keratocytes. This suggests that EAAT 4 may be associated with the keratocytes and potentially play a role in glutamate uptake into these cells.

**Epithelium**

All of the EAAT isoforms were detected in the epithelium (Figure 5). However, there were subtle differences in the labelling patterns for each isoform within the superficial (S), wing (W) and basal (B) cells. EAAT 1, EAAT 3 and EAAT 4 label the superficial, wing and basal layers of the epithelium (Figure 5B, D & E) with membrane labelling evident in the wing and basal cells (Figure 5B, D & E). EAAT 3 and 4 labelling was also detected in the membranes of the superficial cells (Figure 5D & E), whereas EAAT 1 labelling in this layer appeared more cytoplasmic (Figure 5B). EAAT 2 labelling was punctate with membranous labelling detected in the basal layer (Figure 5C). EAAT 5 labelling was uniformly present through all the cell layers and appeared to be associated with the cytoplasm rather than the membranes (Figure 5F).
Taken together, these results demonstrate EAAT 1-5 isoforms to all localise to the corneal epithelium. While the membranous labelling of cells in the basal and wing cell layers for EAAT 1-4 indicates that these cells source their glutamate from the stroma, the membranous labelling for EAAT 3-4 in the superficial cell layers suggests that these cells source glutamate from the tear fluid.

(ii) Glycine transporters: GLYT 1 and GLYT 2

L-glycine is the terminal precursor amino acid required for GSH synthesis and is added to the C-terminus of \( \gamma \)-glutamylcysteine via the enzyme glutathione synthetase to form GSH (Reddy, 1971). L-glycine uptake is mediated by the \( \text{Na}^+ \)-dependent GLYT family of transporters, of which, only two isoforms exist, GLYT 1 and GLYT 2. RT-PCR revealed both GLYT 1 and GLYT 2 to be present at the transcript level (Figure 6A) and western blotting confirmed GLYT 1 and GLYT 2 to also be expressed at the protein level (Figure 6B).

Using the same criteria employed for the EAAT isoforms, we were able to identify specific bands for GLYT 1 and GLYT 2 in the brain and cornea (Table 3). Similar to that seen for the EAATs, it appears that the GLYT s undergo tissue-specific processing in the brain and the cornea, resulting in multiple bands that are of different masses to the predicted molecular mass. A search of the literature, indicates that the variations in molecular masses are most likely a result of differential glycosylation (Table 3).
Having shown that both GLYT 1 and GLYT 2 are expressed at the protein level, we next examined their localisation in the cornea (Figure 7). GLYT 1 was observed to label the epithelium, but not the stroma or endothelium (Figure 7B), while GLYT 2 labelled the epithelium and stroma (Figure 7C). The labelling patterns for the GLYT isoforms in the stroma and epithelium were examined at higher magnification and the results presented below:

**Stroma**

GLYT 2, but not GLYT 1, labelled the stroma (Figure 8A) with high magnification images revealing GLYT 2 labelling to mirror the striated arrangement of keratocytes (Figure 8B). This indicates that stromal keratocytes may be able to uptake glycine via GLYT 2.

**Epithelium**

Both GLYT 1 and GLYT 2 labelled the epithelium. However, GLYT 1 labelling was relatively uniform between the superficial, wing and basal cells, with membranous labelling detected in the basal cell layers and a more cytoplasmic distribution in the superficial and wing cells (Figure 9B). On the other hand, GLYT 2 labelling was absent from superficial cells but present in the membranes of wing and basal cells (Figure 9C). Taken together, these results demonstrate GLYT 1 and 2 to both be expressed in the corneal epithelium. However, while labelling of GLYT 2 to the basal and wing cell layers indicates uptake of glycine from the stroma, superficial cells are most likely to rely on glycine uptake from the tear fluids via GLYT 1.
4. Discussion

In this study, we have extended and completed the molecular inventory of GSH precursor amino acid transporters in the cornea (summarised in Table 4). In addition to previous work identifying the cystine/glutamate antiporter, System Xc- (Li et al., 2012), we have now identified the expression of glutamate transporters, EAAT 1-5 and glycine-transporters GLYT 1-2, at the RNA and protein level in the rat cornea. We have also localised each of these amino acid transporters in the different regions of the cornea and shown that all the transporters are localised to the epithelium which is consistent with a role for these transporters in mediating uptake of precursor amino acids for de novo GSH synthesis.

An interesting finding was that while the amino acid transporters were expressed in the epithelium, there were differences in the localisation of these transporters within the different cellular layers of the epithelium. For example, the EAAT isoforms tended to exhibit the highest levels of staining within the basal and wing cell layers of the epithelium. This suggests that L-glutamate levels may vary between the different corneal epithelial cell layers and that L-glutamate may be generally higher in the basal and wing cells than in most superficial epithelial cells. This indicates that EAATs expressed in the basal and wing cell layers take up L-glutamate from the stroma. However, these cells are also rich in mitochondria (Molon-Noblot and Duprat, 1991) and so, L-glutamate generated by glucose metabolism may also act as source of L-glutamate. Interestingly, Langford and colleagues who mapped EAAT expression in the human corneal epithelium, reported EAAT 1-3 to be predominantly expressed in the basal epithelial cells (Langford et al., 2010), which is similar to the rat. However, different patterns were reported for EAAT 4-5 in the human corneal epithelium compared to the rat. In the human
corneal epithelium, EAAT 4-5 were predominantly localised to the superficial wing cell, whereas in the rat corneal epithelium, EAAT 4 was predominantly expressed in the wing and basal layer while EAAT 5 was uniformly expressed in all cell layers. A range of factors could be responsible for these differences in labelling patterns within the epithelium of the rat and human cornea. These include, the effects of using different commercial sources of EAAT antibodies, post-mortem changes in transporter distribution in donor corneal epithelium, age-related differences and other inherent differences that relate to the physiology of the nocturnal rat versus the diurnal human. Therefore, while it appears that the EAATs are localised to the epithelium in both rat and human corneas, it may be that there are some species-specific differences in the localisation of the EAATs to a specific epithelial layer.

The expression of GSH precursor amino acids to the superficial, wing, and basal cells of the corneal epithelium, suggests that these substrates are likely to be sourced from both the tear fluid and the stroma. In the tear fluid, high levels of L-glutamate and L-glycine levels are present, exceeding levels found in the aqueous humour (Nakatsuksasa et al., 2011). As part of its barrier function, the superficial cells of the cornea contain tight junctions and therefore rely on transporter mediated uptake of amino acids from the tear fluid. It therefore seems reasonable to expect to find transporters for these amino acids in the superficial cells of the epithelium. On the other hand, the localisation of these transporters to the basal and wing cells would suggest that these epithelial layers are dependent, for the most part, on the movement of aqueous humour-derived amino acids across the endothelium and the stroma, followed by active uptake by the epithelium. Veltman and colleagues proposed that the corneal stroma acts as a GSH reservoir for the adjacent corneal layers since administration of BSS Plus containing $^{35}$S-GSH into the anterior
chamber resulted in the accumulation of this radioactive thiol in the stroma (Veltman et al., 2004). Since the endothelial cells constitute a “leaky” barrier characterized by incomplete tight junctions, amino acids maybe permitted to enter from the aqueous humour into the stroma, to provide a source of GSH precursor amino acids to basal and wing cells.

While the majority of amino acid transporters were localised to the epithelium, some transporters were also found to label the stroma and/or the endothelium. The detection of EAAT 4 and GLYT 2 in the stroma is indicative of glutamate and glycine diffusion through stromal tissue and potentially uptake into keratocytes. It is known that keratocytes are particularly sensitive to oxidative stress (Chwa et al., 2008) and that the GSH/GSSG ratio is reduced in human keratoconus cells compared to normal human corneal keratocytes (Karamichos et al., 2014), although the mechanism of GSH accumulation in these cells are unknown. Finally, while EAAT 4 was also detected in the endothelium, further investigations revealed it to be localised within the cytoplasm. It would be interesting in the future to determine whether EAAT 4 can be stimulated to insert into the membrane in response to exposure to oxidative stress.

Overall, by combining our findings with previously published work on GSH uptake and synthesis pathways in the rat cornea (Li et al., 2012), we have generated an emerging model of the GSH accumulation pathways in the cornea (Figure 10). From this model, the existence of regional differences in GSH accumulation in the corneal epithelium and endothelium can be seen. It is evident that the GSH precursor amino acid transporters, including the cystine/glutamate exchanger Xc\(^{-}\), responsible for L-cystine accumulation, are localised to the epithelium demonstrating the reliance of the epithelium on the uptake of GSH precursor amino
acids for GSH synthesis. On the other hand, the transporters capable of mediating direct GSH uptake, OAT3 and NADC3, were localised specifically to the endothelium and were absent from the epithelium (Li et al., 2012). These collective findings indicate that GSH is accumulated via two different pathways in the rat cornea; uptake of GSH precursor amino acids for GSH synthesis in the corneal epithelium, and direct uptake of GSH by the endothelium.

Taken together, our findings demonstrate regional differences in GSH accumulation pathways in the epithelium and endothelium regions of the rat cornea. While it is important for similar mapping studies of GLYTs to be translated to humans and for functional studies to be performed, the findings from this work provide insight into the mechanisms that ensure that GSH levels are maintained in the different regions of the rat cornea. In the future, it is hoped that these findings could be used for the development of targeted therapies to enhance GSH levels as a result of depletion of GSH in the cornea due to ageing (Kannan et al., 1999) or oxidative stress induced corneal pathologies such as keratoconus (Saijyothi et al., 2012).

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Figure legends

Figure 1. Identification of EAAT 1-5 at the transcript and protein level in the rat cornea. (A) PCR products were amplified for EAAT 1, 2, 3, 4 and 5 from positive control tissue, either brain (B+) or heart (H+), or total corneal mRNA (C+). In all experiments, no PCR products were observed in control reactions utilising cornea mRNA which lacked the SuperScript®III/RNaseOUT™ enzyme (C-). (B-F) Left hand panel; Western blot depicting the expected (arrowhead) and additional (asterisk) band sizes of EAAT 1 (B), EAAT 2 (C), EAAT 3 (D), EAAT 4 (E) or EAAT 5 (F) protein in the rat brain (lane B), heart (lane H) and cornea (lane C). Right hand panel; Western blot showing the absence of specific bands in the presence of the antigenic control peptide. Bottom panel; β-Actin labelling to indicate equal protein loading between lanes.

Figure 2. Localisation of EAAT 1-5 in the central region of the rat cornea. (A) Labelling with propidium iodide (PI, red) to highlight the different layers of the cornea. TF, tear fluid; AH, aqueous humour; Epi, epithelium; Endo, endothelium. (B-D) Overview images of corneal sections labelled with PI (red) and EAAT 1 (B) EAAT 2 (C), EAAT 3 (D), EAAT 4 (E) or EAAT 5 (F) antibodies (green). Scale bar = 50µm. Insets B-F. Control peptide experiments in which the EAAT antibody is preabsorbed with an excess of its corresponding blocking peptide.
Figure 3. Localisation of EAAT 4 in the endothelium. (A) Saggital section of the cornea focused on the corneal endothelium labelled with PI (red) and the EAAT 4 antibody (green). Scale bar = 20µm. (B) Corneal en face section labelled with phalloidin (red) and the EAAT 4 antibody (green) to visualise the endothelial monolayer. Scale bar = 6µm.

Figure 4. Localisation of EAAT 4 in the stroma. (A) Overview image of the corneal stroma labelled with PI (red) and the EAAT 4 antibody (green). Scale bar = 50µm. (B) High magnification shots of the stroma labelled with PI (red) and the EAAT 4 antibody (green). Scale bar = 20µm.

Figure 5. Localisation of EAAT 1-5 in the epithelium. (A) High magnification shots of the epithelium labelled with propidium iodide (PI, red) to highlight the superficial (S), wing (W) and basal (B) layers of the epithelium. (B-D) Close up images of the epithelium labelled with EAAT 1 (B) EAAT 2 (C), EAAT 3 (D), EAAT 4 (E) or EAAT 5 (F) antibodies. Scale bar = 20µm.

Figure 6. Identification of GLYT 1 and GLYT 2 in the rat cornea. (A) PCR products were amplified for GLYT 1 and GLYT 2 from brain (B+) positive control tissue or total corneal mRNA (C+). In all experiments, no PCR products were observed in control reactions utilising cornea mRNA which lacked the SuperScript®III/RNaseOUT™ enzyme (C-). (B-C) **Left hand panel:** Western blot depicting the expected (arrowhead) and additional (asterisk) band sizes of GLYT 1 (B) or GLYT 2 (C) protein in the rat brain (lane B), and cornea (lane C). **Right hand panel:** Western blot showing the absence of specific bands in the presence of the antigenic control peptide. **Bottom panel:** β-Actin labelling to indicate equal protein loading between lanes.
Figure 7. Localisation of GLYT 1-2 in the central region of the rat cornea (A) Labelling with propidium iodide (PI, red) to highlight the different layers of the cornea. (TF, tear fluid; AH, aqueous humour; Epi, epithelium; Endo, endothelium). (B-C) Overview images of corneal sections labelled with PI (red) and GLYT 1 (B) or GLYT 2 (C) antibodies (green). Scale bar = 50µm. Insets B-C: Control peptide experiments where GLYT 1 or GLYT 2 antibodies are pre-absorbed with an excess of specific blocking peptide.

Figure 8. Localisation of GLYT2 in the stroma. (A) Overview image of the corneal stroma labelled with PI (red) and the GLYT 2 antibody (green). Scale bar = 50µm. (B) High magnification shots of the stroma labelled with PI (red) and the GLYT 2 antibody (green). Scale bar = 20µm.

Figure 9. Localisation of GLYT 1 & GLYT 2 in the epithelium. (A) High magnification shots of the epithelium labelled with propidium iodide (PI, red) to highlight the superficial (S), wing (W) and basal (B) layers of the epithelium. (B-D) Close up images of the epithelium labelled with GLYT 1 (B) or GLYT 2 (C) antibodies. Scale bar = 20µm.

Figure 10. Molecular model of GSH accumulation pathways in the different regions of the cornea. Localisation of System Xc−, the glutamate transporters EAAT 1-5, the glycine transporters GLYT 1-2 and the GSH synthesising enzyme γ-GCS in the epithelium suggests that GSH synthesis is the predominant means by which GSH is accumulated. Although all of the GSH precursor amino acid transporters are present in the epithelium, there are differences in the membrane localisation of transporters and transporter isoforms to the different cell layers. In the
stroma, EAAT 4 and GLYT 2 appear to associate with keratocytes, indicating that these cells
may be able to accumulate glutamate and glycine, respectively. In the endothelium, the GSH
uptake transporters OAT 3 and NaDC3 are present suggesting that GSH uptake from the aqueous
humor is the predominant means of GSH accumulation in these cells.
### Table 1. Primer sets and expected product sizes.

<table>
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<th>Gene Product</th>
<th>GenBank Account No.</th>
<th>Sense Primers (5’-3’)</th>
<th>Antisense Primers (5’-3’)</th>
<th>AT</th>
<th>Product Size (bp)</th>
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<td>EAAT 2</td>
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<tr>
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<td>30 kDa - not reported</td>
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<td>56 kDa - deglycosylated EAAT 1 (Conradt et al., 1995; Schulte and Stoffel, 1995)</td>
<td>56 kDa – deglycosylated EAAT 1 (Conradt et al., 1995; Schulte and Stoffel, 1995)</td>
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<td>EAAT 2</td>
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<td>55 kDa – deglycosylated EAAT 2 (Bauer et al., 2010; Gebhardt et al., 2010)</td>
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<td>75 kDa - glycosylated EAAT 2 (Grunewald et al., 1998; Kalandadze et al., 2004)</td>
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<td>EAAT 3</td>
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<td>50 kDa - deglycosylated EAAT 4 (Hu et al., 2003)</td>
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<td>75 kDa - not reported</td>
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**Table 2.** Comparison of the molecular mass of EAATs in rat brain, heart and cornea. The predicted molecular mass reported was obtained from The Universal Protein Resource (UniProt consortium, 2015). Observed bands masses are included only for bands which were shown to be specific for a particular EAAT isoform in control peptide experiments. Observed masses which were different to that of the predicted molecular mass were investigated to determine whether similar masses had previously been reported in the literature.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Predicted molecular mass (kDa)</th>
<th>Observed molecular mass (kDa)</th>
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<td>Control (Brain)</td>
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<td>GLYT 1</td>
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<td>30, 33, 37, 46 - kDa - deglycosylated GLYT1 (Lim et al., 2007; Lopez et al., 2005; Zafra et al., 1995)</td>
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<td>60, 65 kDa - glycosylated GLYT1 (Lim et al., 2007; Lopez et al., 2005; Zafra et al., 1995)</td>
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<td></td>
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<td>100 kDa – not reported</td>
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<tr>
<td>GLYT 2</td>
<td>87.8</td>
<td>20-25 kDa – not reported</td>
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<td>65, 70, 85 kDa - glycosylated GLYT2 (Lim et al., 2007; Lopez et al., 2005; Zafra et al., 1995)</td>
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**Table 3. Comparison of the molecular mass of GLYTs in rat brain and cornea.** The predicted molecular mass reported was obtained from The Universal Protein Resource (UniProt) (Consortium, 2015). Bands sizes included are only for bands which were shown to be specific for a particular GLYT isoform in control peptide experiments.
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<tr>
<th>Substrate</th>
<th>Transporter</th>
<th>mRNA level</th>
<th>Protein level</th>
<th>Localisation</th>
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<tr>
<td>L-Cystine*</td>
<td>System Xc- (Li et al., 2012)</td>
<td>✓</td>
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<td>L-Glutamate</td>
<td>EAAT 1</td>
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<td>+</td>
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<td>EAAT 2</td>
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<td>✓</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EAAT 3</td>
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</tr>
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<td>L-Glycine</td>
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<td></td>
<td>GLYT 2</td>
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</table>

Table 4. Summary of transporters involved in GSH synthesis in the rat cornea. ✓ = present; ✓ = absent; - = absent staining; + = low level of staining; ++ = moderate levels of staining; +++ = intense levels of staining; Epi, corneal epithelium; S, superficial epithelial layer; W, wing cell layer; B, basal cell layer; Endo, corneal endothelium. *the oxidised form of cysteine.
Highlights

- Identified glutathione (GSH) precursor amino acid transporters in the rat corneal epithelium
- In contrast to the previous identification of GSH uptake transporters in the rat corneal endothelium
- Indicates regional differences in GSH accumulation pathways in the epithelium and endothelium regions of the rat cornea
- Information may be used in the development of targeted therapies to enhance GSH levels in specific regions of the cornea.