Evoked Expression of the Glutamate Transporter GLT-1c in Retinal Ganglion Cells in Human Glaucoma and in a Rat Model

Robert K. P. Sullivan,1 Elizabeth WoldeMussie,2 Lauren Macnab,1 Guadalupe Ruiz,2 and David V. Pow1

PURPOSE. Glaucoma is a common disease of the eye, a key characteristic consequence of which is the death of retinal ganglion cells. The cause of this loss is unknown, though glutamate-mediated toxicity has been implicated. Glutamate transporters are key regulators of glutamate; therefore, the purpose of the study was to determine whether unusual excitation is associated with unusual expression of one or more transporters.

METHODS. The expression of a splice variant of the glutamate transporter GLT-1 (EAAT2) was examined in normal and glaucomatous retinas from humans and rats.

RESULTS. In normal eyes of humans and rats, GLT-1c was expressed only in photoreceptors. In glaucoma, there was additional robust expression of GLT-1c in retinal ganglion cells, including occasional displaced ganglion cells. Conversely, cells such as displaced amacrine cells and amacrine cells were unlabeled.

CONCLUSIONS. The induction of GLT-1c expression by retinal ganglion cells supports the notion that an anomaly or anomalies in glutamate homeostasis may be evident in glaucoma and that such anomalies selectively influence retinal ganglion cells. By analogy to in vitro experiments in which elevated glutamate levels induce expression of glutamate transporters, the authors hypothesize that expression of GLT-1c may represent an attempt by retinal ganglion cells to protect themselves against elevated levels of glutamate. Such anomalies in glutamate levels cannot be restricted to the ganglion cell layer, as this would not have affected displaced ganglion cells. GLT-1c may be a useful indicator of the extent of stress of the retinal ganglion cells and thus a tool for examining outcomes of potential therapeutic and experimental interventions. (Invest Ophthalmol Vis Sci. 2006;47:3853–3859) DOI:10.1167/iovs.06-0231

Glaucoma, particularly open-angle glaucoma, is the second most common cause of blindness worldwide, and affects almost 2% of the population older than 40 years.1 The key features of causation and consequence have been difficult to disentangle,2 but it is commonly assumed that an elevation of intraocular pressure (IOP) evokes a variety of consequential events, including reduction in blood flow, leading to a partial ischemic insult.3 Such ischemic insults may evoke uncontrolled glutamate release and thus glutamate-mediated toxicity via N-methyl-D-aspartate (NMDA) and other glutamate receptors. This could account for the known selective loss of retinal ganglion cells, due to expression of NMDA receptors at high levels on the retinal ganglion cells. Despite the attractiveness of the hypothesis, there has been significant ambiguity in the data. Although early data supported the notion of abnormal glutamate homeostasis as evinced by elevated vitreous levels of glutamate and a downregulation of the Müller cell glutamate transporter GLAST (also called EAAT1),4–7 this finding has generally not been replicated in further studies of rats, monkeys, and humans.8–12 These ambiguities and disparities may reflect a variety of technical issues such as problems in sampling the disease state at early time points (when causal events may manifest themselves) rather than at later time points (where consequential pathologies states may predominate). Despite these ambiguities, it is clear that therapies that target glutamate receptors, such as the use of memantine, have clear beneficial outcomes in terms of retarding or preventing retinal ganglion cell loss.13,14 Given the robustness of the latter findings, which strongly support a role for glutamate in killing retinal ganglion cells,15 we re-examined the expression of glutamate transporters in glaucomatous eyes. Although the Müller cell transporter GLAST has frequently been examined, because it is probably the dominant retinal glutamate transporter,16–18 there are other glutamate transporters in the retina including GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5 (see Rauen et al.19 for a summary). Studies on retinas with transiently raised IOP20 failed to show any loss of EAAT1, -2, -3, or -5 that could account for retinal ganglion cell loss. EAAT4, which is probably restricted to retinal astrocytes21 has not been studied to date. Despite the apparent elimination of these EAATs as causal or consequential factors in the pathogenesis of glaucoma, some possibilities still exist, due to the presence of multiple splice variants for the transporters EAAT1 and EAAT2, which may not be effectively detected or differentiated by most antibodies. This study focuses on EAAT2.

GLT-1 (EAAT2) exists in several distinct forms, including the originally described form, which we refer to as GLT-1α, along with GLT-1b (also called GLT1v) and GLT1c.22,19 GLT-1c appears to be associated mainly with a population of amacrine cells, whereas GLT-1b/v is associated with cone photoreceptors and populations of bipolar cells.22,23 GLT-1c is normally only expressed by the photoreceptors in the mammalian retina.19 The distribution or levels of expression of GLT-1b/v and GLT-1c have never been examined in glaucomatous eyes. In this study,
we examined whether the expression of the glutamate transporter splice variant GLT-1c might be changed in response to glaucoma, in humans and in a rat model of glaucoma.

**Materials and Methods**

**Elevation of IOP in Rats**

Experiments were conducted in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and strict adherence to internal animal care and use committee guidelines. Male Brown Norway rats weighing 350 to 450 g were used (n = 20). IOP was elevated by laser photocoagulation with blue-green argon laser (Coherent, Palo Alto, CA). Rats were anesthetized with a mixture of ketamine (15 mg/kg), acepromazine (1.5 mg/kg), and xylazine (0.5 mg/kg). Laser treatment was performed on limbal and episcleral veins. Treatment was performed in two parts, one week apart. The amount of energy used was 0.3 W for 0.2 seconds, delivering a total of approximately 0.3 W/s. Animals were then maintained under normal animal house conditions for 2 months.

**Measurement of IOP**

IOP was measured with a handheld tonometer (Tono-Pen; Mentor, Norwell, MA), as described previously.24 Briefly, rats were injected with acepromazine, 3.0 mg/kg intramuscularly, enough to keep them calm but not to sedate them. Proparacaine 0.5% was applied topically to the eyes to anesthetize the cornea. Fifteen readings were averaged to give one measurement. Under this mild anesthesia, IOP in normal eyes is approximately 16 mm Hg.24 IOP measurements are detailed in Table 1.

Control animals were handled in a similar manner, including anesthesia and IOP measurements, but did not receive laser lesions.

**Human Tissues**

Donated human postmortem ocular tissues were obtained as fixed tissues (4% formaldehyde fixation) from the Lions Eye Banks in Brisbane and Sydney and the Lions Eye Institute for Transplant and Research (Tampa, FL). The research adhered to the tenets of the Declaration of Helsinki for biomedical research involving human subjects. Adult human retinas (four normal) were dissected from the Lions Eye Banks. Some of the immunolabeled sections were subsequently counterstained with the fluorescent dye 4',6'-diamino-2-phenylindole (DAPI; Sigma-Aldrich), to label the nuclei of all cells selectively.

**Immunocytochemistry**

Immunocytochemical labeling was subsequently performed according to standard immunoperoxidase or immunofluorescence protocols. The GLAST antibody was used at 1:20,000 for immunoperoxidase labeling. The GLT1c antibody was used at a dilution of 1:5,000 for immunoperoxidase studies and at 1:2,000 for immunofluorescence. The mouse monoclonal MAP-1 antibody and the guinea pig GP611 antibody were used at 1:1,000 for immunofluorescence studies. Immunoperoxidase labeling used biotinylated anti-rabbit antibodies and streptavidin-biotin–HRP complex (both from Amersham, Sydney, NSW Australia) at a dilution of 1:300, and the labeling was revealed using DAB (3,3′-diaminobenzidine) as a chromogen. Immunofluorescence labeling was performed with species-specific anti-Rabbit, anti-mouse, or anti-guinea pig antibodies (Amersham or Sigma-Aldrich), coupled to FITC or Texas red. Some of the immunolabeled sections were subsequently counterstained with the fluorescent dye 4',6'-diamino-2-phenylindole (DAPI; Sigma-Aldrich), to label the nuclei of all cells selectively.

**Results**

**Rat Immunoperoxidase Labeling**

Our immunocytochemical results using the antibody against GLAST revealed that in the pigmented Brown Norway rats, the right eye had been laser lesioned to cause an elevation of IOP. Data are mean mm Hg ± SEM.

**Table 1. IOP Measurements of Left and Right Eyes of Rats over Time**

<table>
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<tr>
<th>Time Point (d)</th>
<th>Right eye</th>
<th>Left eye</th>
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<tr>
<td>0</td>
<td>15.3</td>
<td>14.9</td>
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<tr>
<td>SEM</td>
<td>0.262</td>
<td>0.37</td>
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<tr>
<td>7</td>
<td>21.1</td>
<td>16.6</td>
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<tr>
<td>SEM</td>
<td>0.76</td>
<td>0.44</td>
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<td>14</td>
<td>31.4</td>
<td>15.1</td>
</tr>
<tr>
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<td>0.55</td>
<td>0.41</td>
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<tr>
<td>28</td>
<td>28.9</td>
<td>15.4</td>
</tr>
<tr>
<td>SEM</td>
<td>0.37</td>
<td>0.47</td>
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<tr>
<td>42</td>
<td>27.9</td>
<td>15.2</td>
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<tr>
<td>SEM</td>
<td>0.6</td>
<td>0.49</td>
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<td>58</td>
<td>23.6</td>
<td>14.8</td>
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<tr>
<td>SEM</td>
<td>0.76</td>
<td>0.24</td>
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**Antibodies**

Antibodies used in this study against GLT-1c, which is a splice variant of the glutamate transporter GLT-1 (EAAT2), and the Müller cell glutamate transporter GLAST (EAAT1) were previously raised in this laboratory.17,19-22,25-26 The GLT-1c antibody has been extensively tested in a variety of systems, including the demonstration of its ability to detect GLT-1c but not other forms of GLT-1 in transfected cells, in Western blot analysis it detects a single band at the correct molecular weight, and preabsorption and other standard tests confirm its specificity.34 A mouse monoclonal antibody against MAP1 (Sigma-Aldrich, Castle Hill, NSW, Australia), which is a marker of retinal ganglion cells,27,28 was used to confirm the identity of GLT1c-immunoreactive cells as ganglion cells. An additional antibody (GP611)—which was generated in this laboratory in a guinea pig against an unknown retinal antigen but which appears, fortuitously, to be a useful illustrative staining tool, as it selectively immunolabels the somata of all neurons in the human retina except retinal ganglion cells—was also used to illustrate the differential localization of GLT-1c.

**Table 2. Age and Gender of Normal Human Retinas without Evidence of Retinal Disease**

<table>
<thead>
<tr>
<th>Age (y)</th>
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<th>69</th>
<th>73</th>
<th>85</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>68</td>
<td>Female</td>
<td>69</td>
<td>Male</td>
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</table>

**Table 3. Age and Gender of Human Donor Retinas with Evidence of Glaucoma**

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Gender</th>
<th>70</th>
<th>71</th>
<th>74</th>
<th>76</th>
<th>81</th>
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<tbody>
<tr>
<td></td>
<td>Female</td>
<td>70</td>
<td>Male</td>
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<td>Male</td>
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<td>Female</td>
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</tbody>
</table>
GLAST labeling was abundant in the Müller cells (Fig. 1A), and there was no obvious demonstrable decline in the intensity of immunolabeling for GLAST in response to raised IOP; instead the intensity of the immunoreactivity was indistinguishable from that of controls, or possibly slightly more intense in some retinas (Fig. 1B).

In accord with our previous results, immunoreactivity for GLT-1c was restricted to the photoreceptors (Fig. 2A). Minor variations were observed in intensity of labeling of photoreceptors between animals, but no evidence was found for immunoreactivity in the retinal ganglion cell layer or elsewhere.

By contrast, in all rats that had been subjected to increased IOP, we noted not only the expression of GLT-1c in the photoreceptors, but also strong labeling in cellular elements in the ganglion cell layer across the entire retina (Figs. 2B-E). Labeling appeared to be associated with ganglion cells. Thus, at the optic nerve head and adjacent areas there was clear evidence for axonal labeling in the ganglion cell/nerve fiber layers, with labeling extending into the optic nerve (Fig. 2B). Slightly more peripherally, where the density of axon bundles was lower, individual labeled cell bodies were observed (Fig. 2C). At higher magnification (Fig. 2D) multiple dendrites could be observed, extending from the labeled soma into the inner plexiform layer. Labeling appeared to be predominantly in the plasma membranes of these cells. Similar labeled cell bodies were observed out to the extreme periphery of the retina (Fig. 2E).

**Human Tissues**

Examination of retinas from human eyes where there was no apparent disease (Fig. 3A) revealed, as anticipated from our previous studies, that GLT-1c was expressed in photoreceptors but absent from other cells in the retina. By contrast, in all the retinas from eyes with a clinical diagnosis of glaucoma, the labeling pattern was very different. The pattern of labeling for GLT-1c in human retinas with glaucoma was similar to that observed in rat eyes subject to raised IOP. Immunoperoxidase labeling revealed that at all retinal eccentricities, retinal ganglion cells were weakly labeled for GLT-1c, as were a few rare, large, displaced ganglion cells (Fig. 3B). In many cells, labeling was associated with the plasma membranes of the cells, including the dendrites, and with axon bundles in the ganglion cell/nerve fiber layers (Fig. 3B). Comparison with confocal images of immunofluorescence labeling revealed an entirely comparable pattern of labeling, with most labeling being in the plasma membranes in many cells (Fig. 4A), whereas in other cells there was additional punctate labeling in the cytoplasm (Fig. 4B), suggestive of internalization or trafficking of the transporter in such cells.

**Double Labeling**

To determine whether all or only some of the cells in the ganglion cell layer expressed GLT-1c in response to glaucoma or raised IOP, some sections were counterstained with the fluorescent nuclear stain DAPI. In the rat retina (Fig. 5A), DAPI staining suggested that somata with evidence of GLT-1c labeling accounted for only between a third and half of the cells in the ganglion cell layer, suggesting that the displaced amacrine cells were not labeled. Similar results were obtained for human retinas from eyes with glaucoma (Fig. 5B). Confirmation of this pattern of labeling of ganglion cells for GLT-1c, but not the smaller displaced amacrine cells, was provided by double labeling with a guinea pig antibody GP611 that appears to label somata of all retinal neurons apart from those of retinal ganglion cells. Double labeling showed clearly that the population of cells in the ganglion cell layer that expressed GLT-1c were not immunolabeled with GP611, but the GP611 antibody strongly labeled somata of small cells in the GCL, which we interpret as being the somata of displaced amacrine cells. Finally, confirmation that the cells that express GLT-1c were indeed ganglion cells, was demonstrated by double labeling for Map-1. The GLT-1c-immunoreactive cells were also immunoreactive for MAP-1 in both rat (Fig. 5D) and human (Fig. 5E) retinas.

**DISCUSSION**

In this study, we demonstrate that retinal ganglion cells that express the retinal ganglion cell marker MAP-1 showed the capacity to respond to raised IOP by expressing a specific glutamate transporter, GLT-1c, that is normally only expressed by photoreceptors. The selectivity of this expression in ganglion cells alone suggests that the stimulus that evokes this expression may be specific for these cells alone. Moreover, the comparable expression in both human and rat tissues suggests that raised IOP may activate similar physiological responses in both species.

The specific pattern of expression is clearly of interest, since it is the retinal ganglion cells that selectively die in glaucoma. Our data thus support the idea that there is some perturbation of the glutamate system either upstream of or directly involving the retinal ganglion cells, that evokes the induction of GLT-1c expression, possibly as part of a homeo-
static mechanism. In support of this view, it has been shown that expression of GLT-1 is dependent on glutamatergic transmission, inferring that there is a homeostatic link between glutamate transporter expression and extracellular levels of glutamate. In the brain, it has been shown that loss of glial glutamate transporters after an hypoxic insult, which causes an elevation of extracellular glutamate, also evokes a consequent rapid switch on of a splice variant of GLT1 (GLT-1b) that extended multiple dendrites into the inner plexiform layer. We propose that the switching on of GLT-1c in retinal ganglion cells that we observe in this study may represent a similar response to elevated extracellular glutamate. This suggestion appears to agree with other functional data. Thus, careful perusal of the data of Barnett and Grozdanic shows that 25 days after an insult by transient elevation of IOP, n-aspartate, which is a substrate for all glutamate transporters, appears to be accumulated by rat ganglion cells, as well as the Müller cells, whereas no accumulation in ganglion cells is evident in the control results presented. This observation is in accord with the notion that ganglion cells may activate the expression of GLT-1c after an insult.

The evidence that we present for an anomalous expression of a glutamate transporter is novel and is a potential indicator of those cells, which are under stress and are likely to subsequently die. The data are most readily interpreted in the context of the aberrant glutamate homeostasis hypothesis and are in accord with recent findings showing that drugs such as...
memantine, which act as NMDA receptor antagonists, can block the death of retinal ganglion cells in both rats and monkeys, an observation that implies abnormal activation of such receptors in the first instance.

It is widely accepted that the predominant glutamate transporter in the retina is GLAST, which is expressed by Muller cells. Our observations with respect to GLAST suggest either no change, or a very modest increase in GLAST expression in response to an increase in IOP. This agrees entirely with previous observations in albino rats in the same model, where a modest increase was reported at 1 month after laser lesioning, followed by a return to normal levels by 2 months. One possible explanation for this conundrum may be evident in the studies by Susarla et al., who have shown that apparent loss of GLAST immunoreactivity in astrocyte cultures is associated with increased glutamate transport, a paradoxical finding that has been interpreted by these workers as indicating that immunocytochemically detectable epitopes may be modified by changes in phosphorylation, yielding a form of GLAST that has enhanced glutamate transport capacity but cannot be detected by antibodies. Accordingly, in glaucoma, stable levels of immunocytochemically detectable GLAST or a slight increase in GLAST could be associated paradoxically with lower levels of glutamate transport. This association remains to be determined.

Although our results strongly support the notion that the expression of GLT-1c may be a diagnostic marker of retinal ganglion cells that are stressed in glaucoma, we do not know whether the expression of GLT-1c is directly induced by glutamate, or whether there are additional mechanisms involved. A series of downstream experimental possibilities exist, including attempts to modulate the expression of GLT-1c by admin-

**Figure 3.** Human retinas immunoperoxidase labeled for GLT-1c. (A) A normal human retina (male aged 68) exhibited labeling only in the photoreceptor layer (P). In a retina (from a male aged 71) with a diagnosis of glaucoma (B) retinal ganglion cells (arrows) and a displaced ganglion cell (arrowhead) were labeled, as were nerve fiber bundles. Scale bars, 50 μm.

**Figure 4.** Retinas from the human eye (male aged 81) with glaucoma, immunofluorescence-labeled for GLT-1c. (A) Strong labeling was observed in photoreceptors (P), particularly the synaptic terminals. Strong labeling was also present in the plasma membranes of ganglion cells. (B) Some ganglion cells (G) exhibited immunoreactivity in intracellular inclusions (arrow) rather than in the plasma membranes, indicative of GLT-1c being trafficked to or from the plasma membranes. Scale bars, 20 μm.
istration of glutamate, NMDA, or memantine, and the experimental manipulation of GLAST activity using antisense knockdown. These determinations, however, were outside the scope of the present study.

References


17. Pow DV, Barnett NL. Changing patterns of spatial buffering of glutamate in developing rat retinas are mediated by the Muller cell glutamate transporter GLAST. Cell Tissue Res. 1999;297:57–66.