Changes of retinal glutamate transporter GLT-1 mRNA levels following optic nerve damage

Christian Mawrin,1 Thomas Pap,2 Martina Pallas,3 Knut Dietzmann,1 Wolfgang Behrens-Baumann,3 Christian K. Vorwerk3

Departments of 1Neuropathology, 2Rheumatology, and 1Ophthalmology, Otto-von-Guericke-University, Magdeburg, Germany

Purpose: Under physiological conditions, levels of the excitatory neurotransmitter glutamate within the retina are regulated by retinal glutamate transporters to prevent toxic accumulation. Alterations in this glutamate buffering have been implicated in retinal ganglion cell (RGC) death. We quantified the changes in the level of glutamate transporter mRNA in a model of acute rat optic nerve injury.

Methods: Optic nerve damage was induced in one eye of 25 adult Wistar rats by partial optic nerve crush (ONC). Total mRNA levels of the retinal glutamate transporter GLT-1 (EAAT-2) were determined by quantitative real-time PCR. GLT-1 mRNA levels were measured 1, 3, 7, 14, and 28 days following optic nerve injury. Additionally, control values were obtained from the retinas of five control rats (sham-crush).

Results: In the very early phase (1 day post-ONC), a 3.9-fold increase in levels of GLT-1 mRNA was observed in the ONC retinas compared with control eyes. This was followed by a rapid decrease towards control levels at day 3 post-ONC. GLT-1 mRNA levels remained up to 14 days post-crush. However, in the late phase post-ONC (day 28), the level of GLT-1 mRNA increased again, but still remained not significant to control levels.

Conclusions: Changes in GLT-1 mRNA expression following axonal trauma of RGCs can lead to an imbalance of glutamate homeostasis. This may cause local accumulation of toxic concentrations of the neurotransmitter glutamate and further irreversible excitotoxic damage of RGCs.

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system. Within the retina, glutamate is released by photoreceptors, bipolar cells, and ganglion cells [1,2]. Under normal conditions, extracellular glutamate is rapidly transported into the intracellular space by glutamate transporters, resulting in maintenance of the physiologic glutamate concentration [3].

However, in addition to its normal action in the retina, increased glutamate levels can result in toxic damage of retinal ganglion cells (RGC). It has been shown that an “excitotoxic” pathway, mediated primarily through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, contributes significantly to RGC damage [4-6], with the experimental administration of an NMDA antagonist shown to prevent glutamatergic toxicity [7].

It has been suggested that increased extracellular glutamate levels may be due, at least in part, to a failure of glutamate transporter buffering [8]. Among the five excitatory amino acid transporters identified to date, four are found in the retina. EAAT1 (Excitatory Amino Acid Transporter; GLAST) is found in Müller cells and astrocytes [9]. EAAT2 (GLT-1) is localized to cones and two types of bipolar cells [10]. EAAT3 (EAAC1) is found on horizontal, amacrine, and ganglion cells, and occasionally on bipolar cells [11]. EAAT5 is localized to photoreceptors and bipolar cells [12].

Previous studies using immunohistochemistry have demonstrated a reduced expression of EAAT-1 in a rat glaucoma model [13]. It has been shown in rats that treatment with antisense-oligonucleotides to GLT-1 leads to increased vitreous glutamate levels and RGC death [14]. In addition, in human glaucoma is the expression of this glutamate transporter reduced at the protein level [15]. Following experimental glaucoma induction in rats, with subsequent optic nerve damage, Martin et al. [16] recently found no change in the expression of GLT-1 by immunohistochemistry. However, Western blot analysis revealed a significant decrease in the levels of GLT-1 protein. Interestingly, following optic nerve transection, which leads to extensive RGC death, the GLT-1 protein levels were found to be increased in this study [16].

These findings suggest that alterations in glutamate transporter levels in different animal models of optic nerve injury may be determined primarily by the time course of the retinal damage. In the optic nerve crush (ONC) model, rapid primary and secondary damage to RGCs has been shown. In models of chronic retinal damage, as seen following prolonged increase of intraocular pressure, all retinal cells (glial and neuronal) as well as the optic nerve itself are affected [16]. However, in models with rapid RGC damage, it is important to determine whether the buffering function of the glutamate transporters, which occurs in response to the increased glutamate levels, may be caused by the activation of stored glutamate transporter proteins from retinal cells. Alternatively, this may be due to de novo protein synthesis following rapid transcription of glutamate transporter mRNA.
In the present study, we describe the quantification of GLT-1 mRNA over a 4 week period following rapid optic nerve injury. We report a biphasic pattern of GLT-1 mRNA levels with a rapid increase in the early phase following optic nerve damage, and a downregulation towards control level with time.

METHODS

Animals and surgery: All procedures were performed in accordance with the ARVO statement of the Use of Animals in Ophthalmic and Vision Research and the animal care guidelines published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals). A total of 30 Wistar rats (Harlan-Winkelmann, Hannover, Germany) were housed in a 12 h light/dark cycle with water and food ad libitum. At the time of ONC, rats weighed between 250 g and 300 g.

Anesthesia was obtained with intraperitoneally administered chloral hydrate (6 ml of a 7% solution per kg body weight). The optic nerve was approached from the orbit by incision of the conjunctiva under the guidance of a surgical microscope. The retractor bulbi muscle was separated, and the optic nerve was exposed by blunt dissection, leaving both retinal blood supply and dura intact. The nerve was then crushed at a distance of approximately 2 mm from the eye cup for 30 s using self-closing Castroviejo cross-action forceps (model 35-315-10; Martin Instruments, Tuttlingen, Germany), which were modified and calibrated as previously described [17]. Only one optic nerve was crushed in each animal. However, the contralateral eye was not used as a control because unilateral optic nerve damage may result in altered gene expression in the contralateral eye [18]. For control purposes, five rats were sham-crushed by performing the operating procedure without closing the crush forceps and damaging the optic nerve.

Rats were euthanized by chloral hydrate overdose 1, 3, 7, 14, and 28 days post-ONC. Five animals were analyzed at each time point. The eyes were enucleated within seconds of death, the retinas were dissected, and immediately snap-frozen in liquid nitrogen; the retinal tissue was stored at -80 °C. All rats were euthanized approximately 4 h after onset of the light period. This procedure precluded differential influences of the light/dark cycle on retinal gene expression, which have previously been reported [19].

Quantitative real-time PCR: For each experiment, total RNA was extracted from retinal tissue using the TRIZOL® kit according to the manufacturer’s instructions (Life Technologies, Karlsruhe, Germany) and reverse transcribed with random hexamer primers. Following RNA isolation and cDNA synthesis, the quality of the cDNA was controlled by PCR using rat specific primers for β-actin. Amplification of the expected PCR product was successful in all samples (data not shown).

The level of GLT-1 mRNA was analyzed by quantitative real-time PCR using a fluorogenic 5'-nuclease assay (TaqMan®, Applied Biosystems, Weiterstadt, Germany) on an ABI Prism 7900 HT Sequence Detection system. For quantitative real-time PCR, primers and probes were applied as follows: Forward primer: 5'-AGT CCG AGC TGG ACA CCA TT-3'; Reverse primer: 5'-ACG GAC TGC GTC TTG GTC AT-3'; TaqMan Probe (FAM-TAMRA labeled): 5'-CAA CAC CGA ATG CAC GAA GAC ATC GA-3'.

It is not known whether these primers span introns. To minimize the potential contamination of RNA samples with genomic DNA, the samples were DNase digested. Standard annealing-extension conditions were used for 40 cycles (denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, extension at 72 °C for 30 s), and 18S rRNA was co-amplified as an internal standard. Data were calculated as x-fold expressions versus 18S rRNA using the DC, method as previously described [20].

Statistical analysis: The Mann-Whitney U test was used to compare the expression levels of GLT-1 in the different groups for statistical significance. A p value less than 0.05 was considered significant.

RESULTS

Histologic examination of retinal cross sections revealed a substantial loss of RGCSs, whereas other layers did not show major differences compared to sham-crushed control animals (Figure 1).

Quantification of the retinal GLT-1 mRNA at various time points revealed that the level of the glutamate transporter increased (3.9 fold) compared with control levels in the very early phase (1 day) post-optic nerve damage (Figure 2). This early increase was followed by a rapid decrease to 38% of control levels on day 3 post-ONC (Figure 2). The reduction in GLT-1 mRNA levels (28% of control) remained on day 7 and 14 following optic nerve injury compared with controls (Figure 2). A slight increase in the level of GLT-1 mRNA was observed on day 28, but remained below control levels (80% of control; Figure 2).

Figure 1. RGC loss in retinal cross sections after ONC. Hematoxilin-eosin stain of a representative cross sections of a retina 14 days post-ONC (A) and a sham-operated control retina (B). A marked reduction in retinal ganglion cells is evident. The retinal ganglion cell layer (RGCL), inner nuclear layer (INL), and outer nuclear layer (ONL) are labeled.
DISCUSSION

Partial ONC is a valuable model that allows the analysis of changes in the levels of excitatory amino acids and their respective transporters in association with RGC death [5,21]. We report here that one of the glutamate transporters involved in intraocular glutamate buffering, GLT-1 (EAAT2), is regulated in a specific manner following experimental induced optic nerve injury, causing rapid RGC death.

In the retina, GLT-1 is localized to cones and bipolar cells [10]. Following acute experimental retinal damage, a rapid increase in intraocular glutamate levels is observed [22]. Using in situ hybridization, retinal GLT-1 mRNA in ischemia induced retinal damage has previously been shown to be increased within 48 h. This is suggested to represent a mechanism to counteract the deleterious effect of raised extracellular excitatory amino acid concentrations on RGCs [23]. If elevated intraocular pressure and subsequent retinal damage persists, as seen in human glaucoma or in animal models of experimental glaucoma, then immunohistochemistry and Western blotting confirms levels of GLT-1 to be reduced [15,16]. However, it is unclear whether the lack of glutamate buffering GLT-1 is due to saturation of the transporter proteins present, or reduced transcription of the GLT-1 mRNA, or possibly a combination of both. Interestingly, in contrast to the repeatedly observed changes in the glutamate transporter, it is unclear whether the intraocular glutamate levels are altered in the long term course of glaucoma. Reports of raised glutamate levels in humans and monkeys [24] have been questioned by recent findings of unchanged glutamate levels in experimental glaucoma [25].

In the present study, we show that previous observations reporting decreased amounts of GLT-1 protein optic nerve damage are caused at least in part by a downregulation of the GLT-1 mRNA after an initial upregulation. However, in the very early phase post-ONC, we measured markedly increased GLT-1 mRNA levels. As ONC leads to rapid and substantial RGC death with subsequent acute retinal glutamate release, this observation can perhaps be explained by a compensatory upregulation of the glutamate transporter. In this context, it has been reported that glutamate release is able to induce the expression of transcription factors such as CREB [26,27], as well as immediate early response genes such as c-fos [28,29] in the rat retina. However, the precise mechanisms of transcriptional regulation of retinal GLT-1 remain unknown.

Following the early upregulation of GLT-1, we found a lasting reduction of GLT-1 mRNA levels compared to the initial increase, beginning day 3 post-optic nerve injury. This observation is in accordance with previous studies reporting a loss of GLT-1 protein, and suggests that transcriptional downregulation contributes significantly to this decrease. As suggested by Martin et al. [16], this sustained decrease of GLT-1 may be the result of decreased synaptic activity from dying glutamatergic neurons, a loss of neurotrophic factors due to a reduced number of neurons in the retina, or primary injury to Müller cells and bipolar cells. However, it does not provide a sufficient explanation for our finding that GLT-1 mRNA levels return to values slightly above those of controls after 28 days, but without reaching statistical significance. Therefore, we propose that the change in the GLT-1 mRNA levels represent a transcriptionally regulated physiologic response to both acute and chronic sublethal injuries in the eye.

ACKNOWLEDGEMENTS

This project was supported by a Start-up-grant to Christian K. Vorwerk from the University of Magdeburg, Germany. The expert technical assistance of Ines Schellhase and Desire Weber is gratefully acknowledged. Furthermore, we thank Dr. Elmar Kirches for critical reading of the manuscript.

REFERENCES


The print version of this article was created on 13 Jan 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.