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The Selective Vulnerability of Retinal Ganglion Cells in Rat Chronic Ocular Hypertension Model at Early Phase

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Abstract Glutamate neurotoxicity has been postulated to play a prominent role in glaucoma. In this study the possible roles of two subunits of glutamate receptors during the early phase of retinal ganglion cell (RGC) loss in a rat chronic ocular hypertension (COH) model were investigated. COH was induced by applying argon laser to the episcleral and limbal veins of the right eye of rats, the observation times were at 4, 14 and 28 days after the first laser. RGCs were retrogradely labeled by putting Fluoro-Gold (FG) on the surface of both side superior colliculus. Immunohistochemical staining using specific antibodies against N-methyl-D-aspartate receptor 1 (NR1) or glutamate receptor 2/3 (GluR2/3) was performed on the retinal sections of normal and COH eyes. Fluorescent images were captured using confocal laser scanning microscope and the number of NR1 and GluR2/3 labeled cells were counted and cell size was measured using Stereo Investigator. During the observation period, the numbers of NR1 and GluR2/3 positive RGCs in the RGC layer were reduced parallel to the loss of RGC. The dramatic loss of GluR2/3 immunoreactive neurons occurred starting immediately after the first laser to 4 days while the dramatic loss of NR1 immunoreactive neurons occurred from 14 to 28 days after the first laser. Size difference was detected in NR1 immunoreactive RGCs, large ones were more sensitive to the high ocular pressure. These results suggest that both NR1 and GluR2/3 are involved in the mediation of RGC death in the early stage of COH.

Keywords Retinal ganglion cell · Ocular hypertension · Glutamate receptor · Selective vulnerability · Retina

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Introduction

Glaucoma is an aging-related neurodegenerative disease associated with irreversible loss of retinal ganglion cells (RGCs) and their axons. It is the second leading cause of blindness worldwide (Resnikoff et al. 2004). Clinically, glaucoma patients have characteristic visual field loss, suggesting that a subpopulation of the RGCs is more vulnerable to injury. Morphologically, large RGCs which are related to the magnocellular pathway are selectively damaged in human and experimental glaucoma in monkey (Glovinsky et al. 1991, 1993; Quigley et al. 1989). However, the mechanism of differential loss is not known yet and glutamate neurotoxicity has been postulated to play a prominent role (Luo et al. 2001; Zhou et al. 2008). The elevated level of glutamate in the vitreous body of glaucomatous eyes was detected in the animal models using rats, monkeys and dogs (Dreyer et al. 1996; Brooks et al.

1997). Both in vivo and in vitro exposure to glutamate or NMDA can cause damage in RGCs (Lam et al. 1999; Zhou et al. 2008), and their toxic effects can be blocked by antagonist of NMDA, MK-801(Chaudhary et al. 1998).

Glutamate exerts its toxic effect on RGCs through the glutamate receptor subtypes. Glutamate receptors are divided into ionotropic and metabotropic (G protein-coupled) receptor subtypes. According to their preferred agonists, ionotropic glutamate receptors are subdivided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors. The latter includes α-amino-3-hydroxyl-5-methyl-isoxazol-4-propionic acid (AMPA) and kainate subtypes. In addition, many subunits of the subtypes have been identified. They are NMDA receptor 1 (NR1), NR2A-D and NR3A-B of NMDA receptors; glutamate receptor 1-4 (GluR1-4) of AMPA receptors; GluR5-7 of kainate receptors; and metabotropic glutamate receptor 1-8 (mGluR1-8) (Hollmann and Heinemann 1994; Nakanishi et al. 1998; Chatterton et al. 2002). All subunits of NMDA and AMPA receptors are distributed in the RGCs and amacrine neurons of the rat retina visualized with immunohistochemistry (Grunder et al. 2000a, b). We hypothesize that the distribution and function of the glutamate receptor subunits (NR1 and GluR2/3) might be involved in the RGC vulnerability at early stage of ocular hypertensive insult.

Using the laser photocoagulation induced rat chronic ocular hypertension (COH) model (Chan et al. 2007; Ji et al. 2004; Li et al. 2006), retrograde labeling and immunohistochemical analysis were used together to detect the temporal change of NR1 or GluR2/3 labeled RGC at early stage of this COH model. Result showed that the dramatic loss of GluR2/3 immunoreactive neurons occurred before that of the NR1 immunoreactive neurons. Loss of NR1 immunoreactive RGCs were positively related with the cell size under COH.

Materials and Methods

Experimental Glaucoma Model

Twenty normal adult female Sprague-Dawley (SD) rats (250–280 g) were obtained from the Laboratory Animal Unit of the LKS Faculty of Medicine, The University of Hong Kong, and were maintained in a temperature-controlled room with a 12 h light/dark cycle. Handling of animals adhered to the ARVO statement for the use of animals in ophthalmic and vision research and was approved by the University of Hong Kong Committee for the Use of Live Animals in Teaching and Research (CULATR). Prior to IOP or any other operation, the rats were anesthetized with an intra-peritoneal injection of a ketamine/xylazine mixture (ketamine 80 mg/kg and xylazine 8 mg/kg; Alfasan, Woerden, Holland). One drop of proparacaine hydrochloride (0.5% alcaine, Alcon-Couvreur, Belgium) was applied to the eyes as a topical anesthetic.

Experimental glaucoma was induced in the right eye of each animal using laser photocoagulation according to our previous publications (Chiu et al. 2007; Ji et al. 2004; Li et al. 2006; Fu et al. 2008). Left eye of each rat did not receive laser treatment and served as contralateral control for IOP. Briefly, the limbal vein and the three radical episcleral aqueous humor drainage veins (superior nasal, superior temporal and inferior temporal) were photocoagulated (power 1,000 mV; spot size 50–100 μm; duration, 0.1 s) using an Argon laser (Ultima 2000SE Argon Laser, Coherent, USA). About 60 laser spots around the limbal vein (except the nasal area) and 15–20 laser spots on each episcleral aqueous humor drainage vein were applied. To maintain high IOP, a second laser treatment at the same setting was applied 7 days later to block any reconnected vascular flow. After each laser treatment, ophthalmic Tobrex ointment (3% tobramyxin, Alcon-Couvreur, Belgium) was applied topically to prevent infection.

Five rats were used as normal control and experimental glaucoma was induced in the right eye of other fifteen rats. The animals survived for 4, 14 and 28 days ($n = 5$) after the first laser photocoagulation. IOP was measured with a Tonopen XL tonometer (Mentor®, Norwell, USA) before and on day 2, 4, 8, 14, 21, 28 after the first laser treatment. To avoid diurnal variation and the effect of anesthesia, all IOP measurements were taken at 10 a.m. and within 15–30 min after anesthesia using a ketamine and xylazine mixture (i.p.). Every time an average of ten records was used to determine the IOP of each eye.

About 7 days prior to euthanization, a small piece of gel foam (Upjohn, Kalamazoo, MI, USA) soaked with 6% Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) was placed over the entire surface of superior colliculus (SC) for retrograde labeling of the surviving RGCs (Chan et al. 2007; Chiu et al. 2008; Ji et al. 2004). Analgesic rimadyl (0.025 mg/ml) was applied in drinking water for 5 days after the surgery. Rats were killed with an over-dose of a mixture of ketamine/xylazine and transcardiac infused with 0.9% normal saline to flush out the blood. Both eyes were enucleated and then post-fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 4°C overnight and then immersed in 30% sucrose. Ten-micron-thick retinal sections containing whole-length retinas and optic nerve heads were cut using a cryostat (CM1900, Leica) and mounted. Three consecutive sections containing optic nerve head were collected from each retina.

Immunofluorescence Staining of Retina

Immunofluorescence histochemistry was carried out following our previous method (Ng and Yung 2001). Briefly, the radial sections of retina were washed with 0.01 M PBS for 5 min and incubated in a mixture of 4% normal goat serum (NGS) and 0.01 M PBS containing 0.1% Triton X–100 for 20 min at room temperature. The sections were incubated in the primary antibodies of NR1 (1:200; Chemicon) or GluR2/3 (1:200; Chemicon) overnight at 4°C. The specificity of antibody was tested by omission of the primary antibody. After washing, the sections were incubated in the secondary antibodies of Alexa 568 (conjugated with anti-rabbit IgG, 1:1,000; Molecular Probe, Invitrogen, Calsbad, USA) for 2 h at room temperature. After thorough washes, the retinal sections were mounted using fluorescent mounting medium (Dako, Glostrup, Denmark) and retinal sections were scanned by a LSM-510 laser scan confocal microscope (Carl Zeiss). Confocal images from four areas in each retinal section from all groups were captured. The four areas are selected from both sides of the optic nerve head at 1,000 μm (paracentral) and 3,000 μm (peripheral) from the central of the optic nerve head. The length of the retina measured in each visual field was 225 μm. The cell size in each image was measured using Stereo Investigator® (MicroBrightField, Colchester, USA).

Statistical Analysis

The cell counts for each marker of all the groups were statistically analyzed with one-way ANOVA and Turkey test. Only cells labeled with FG were counted and therefore, amacrine cells were excluded from the analysis. The percentage of NR1 or GluR2/3 positive RGCs was calculated by obtaining the ratio of number of NR1 or GluR2/3 positive RGCs/total number of FG labeled RGCs. To analyze the correlation of the cell size and cell loss, the data were divided into two groups: normal and glaucoma. SigmaStat® was used to perform the linear regression analysis between cell number and cell size of RGCs in the control and glaucomatous retinas.

Results

Intraocular Pressure

In consistence with our previous study using the same animal model (Chan et al. 2007; Fu et al. 2008; Ji et al. 2004; Li et al. 2006), the average IOP of normal eyes was around 15 mmHg. After photocoagulation using argon laser, the IOP of the right experimental eyes was increased

on the second day after operation and reached the highest level ($\sim$27 mmHg) after the second laser treatment. The IOP was maintained at a high level ($\sim$22 mmHg) for 1 month after the first laser. At the same time, the IOP of the contralateral left control eyes remained at a much lower level ($\sim$15.5 mmHg; Fig. 1a). The IOP of laser treated eyes was significantly elevated compared with those of control eyes (* $P < 0.001$).

Loss of RGC in the Ocular Hypertension Rat Eyes

It is well known that about 98% of RGCs project to the contralateral SC in rat (Forrester and Peters 1967). Applying FG on both side of SC retrogradely labels the RGCs and provides an useful tool for investigating cell survival in the retina. On the flat mounted retina, the
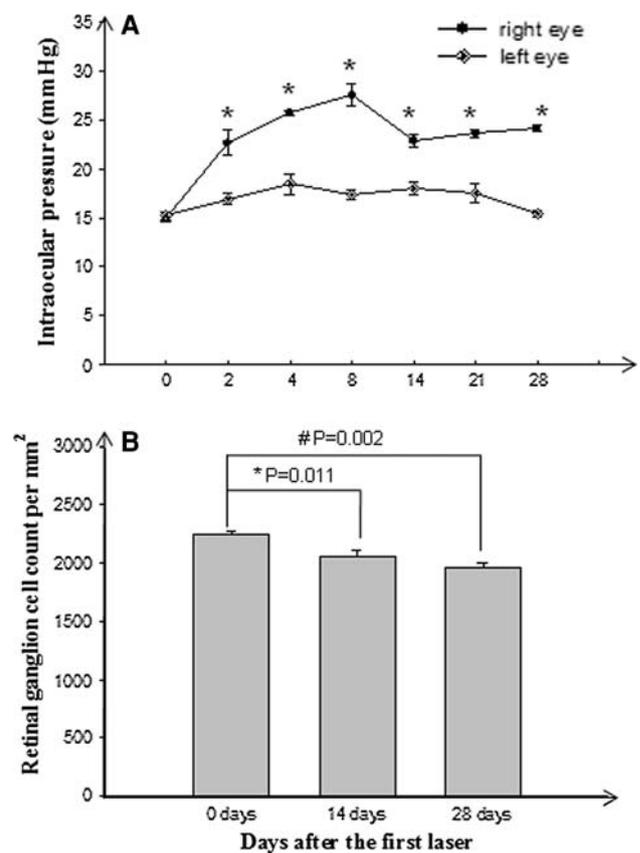

Fig. 1 The changes of IOP and RGCs after the first laser photocoagulation. **a** IOP of the glaucomatous right eyes increased at second day after the first laser and the elevated IOP can be maintained for 1 month after two consecutive laser treatments on the limbal and episcleral drainage veins. IOP of the left eyes remained at normal level. At the same time point, the means of IOP from right eyes compared with that of contralateral left eyes were statistically different (* $P < 0.01$). **b** Density of RGC (per mm^2) significantly decreased after the first laser photocoagulation. Compared with normal retina, there was significant percentage of RGC loss starting from 2 weeks after the first laser photocoagulation (* $P = 0.011$). *Error bars* represent the SEM

density of RGCs in the normal control eyes was $2,241 \pm 27$ cells/mm^2 (Fig. 1b) and this was comparable with our previous reports (Li et al. 2006; Yip et al. 2006). Compared with normal retina, there was significant loss of RGC in the experimental eyes starting at 2 weeks after the first laser treatment (* $P = 0.011$) and there was about 1,964 RGCs/mm^2 left at 1 month after the first laser (#$P = 0.002$, Fig. 1b). The loss of RGC was about 17% of the normal retina.

Same trend of RGC loss in the experimental glaucomatous retina was also observed in the radial retinal sections, there was progressive loss of FG labeled RGC in the retinal ganglion cell layer (RGCL) starting from 4 days and statistically significant from 14 days after the first laser photocoagulation (* $P < 0.05$, Fig. 2a).
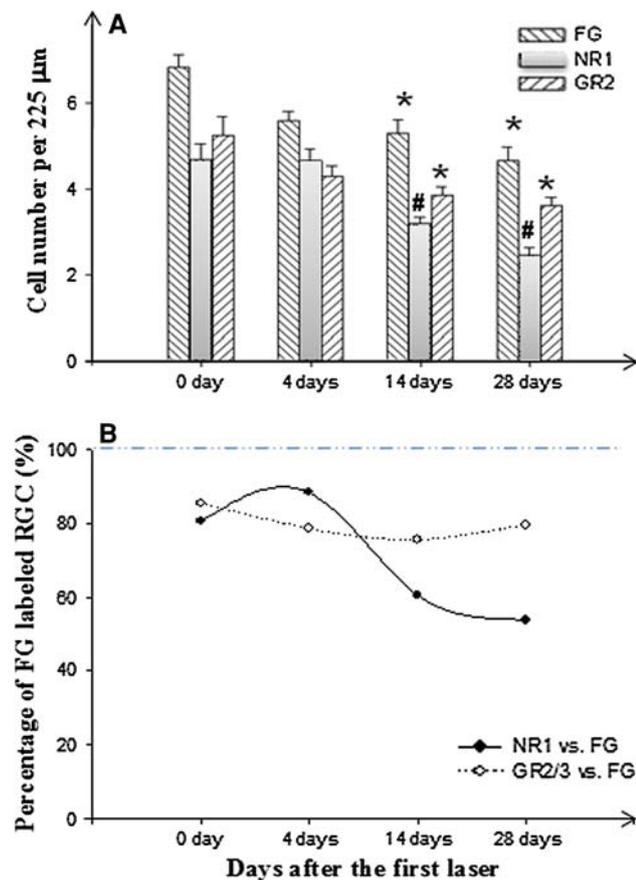
Fig. 2 Effects of ocular hypertension on the survival RGCs labeled with FG, NR1 and GluR2/3. **a** The number of FG labeled RGCs reduced from 4 to 14 ($P < 0.05$) and 28 days ($P < 0.01$). Compared with the normal control, NR1 positive RGCs was significantly decreased in the 14 and 28 days glaucoma ($P < 0.01$); GluR2/3 immunoreactive RGCs was statistically reduced in 14 and 28 days glaucoma ($P < 0.01$). **b** Percentage of NR1 positive RGCs was relatively increased at 4 days after the first laser and then dramaticaly decreased at 14 and 28 days after the first laser. Percentage of Glu2/3 positive RGCs decreased right after the first laser and stabilized since 14 days after the first laser (*spotted line*)

Differential Loss of NR1 or GluR2/3 Labeled RGC in the Ocular Hypertension Eyes

In normal retinal section, about 80% of the FG labeled RGCs expressed NR1 (Figs. 2b, 3c) while about 85% of FG labeled RGCs expressed GluR2/3 (Figs. 2b, 4c). A few of NR1 or GluR2/3 positive neurons in the RGCL that were small in size did not contain FG particles in the cytoplasm, which might be displaced amacrine cells (Figs. 3, 4).

NR1 positive neurons were detected in both the RGCL and the inner nuclear layer (INL) (Fig. 3b, e, h, k, arrowheads). At 4 days after the first laser, the number of NR1 positive neurons in the RGCL was similar to normal retina (Figs. 2a, 3c, f, arrows). NR1 positive RGC was significantly reduced starting from 14 days (#$P < 0.05$, Figs. 2a, 3i, l). When calculated the percentage of NR1 positive FG labeled RGC loss, there was an upregulation at 4 days in the surviving RGCs after the first laser. The dramatic loss of NR1 positive RGCs mainly occurred from 14 to 28 days after the first laser (Fig. 2b).

The similar localization of GluR2/3 immunoreactive neurons could be found in the RGCL and the INL of retina (Fig. 4b, e, h, k, arrowheads). FG and GluR2/3 double labeled RGCs were mainly localized in the small to medium sized neurons in the RGCL (Fig. 4c, f, i, l, arrows). The loss of GluR2/3 positive RGCs started immediately after the first laser (Fig. 2b). In COH retina, the largest reduce in the number of GluR2/3 positive RGCs was from immediate after the first laser to day 4 (Fig. 2) and there was significant loss at 14 days after the first laser (* $P < 0.05$, Figs. 2a, 4i, l). Quantitatively, at 28 days after the first laser, there was an overall 5.8% loss of GluR2/3 immunoreactive RGC and 26.7% loss of NR1 immunoreactive RGC in the COH eye compared with the normal control eye.

Loss of NR1 Immunoreactive RGCs was Size Dependent

In the radial retinal sections the size of FG retrograde labeled RGCs was different, ranging from 50 to 350 μm^2. Most of lost RGCs were larger than 150 μm^2 while the RGCs smaller than 150 μm^2 was relatively increased in the glaucomatous retinas (Fig. 5). NR1 positive large ganglion cells are more sensitive to the elevated IOP. The loss of NR1 positive RGCs was limited to cells larger than 100 μm^2 (Fig. 6). And regressive analysis of NR1 positive RGCs in the glaucomatous retinas showed that correlating factor was 0.76, indicating that there was a positive correlation (#$P < 0.05$) between the loss of cell and cell size. The size of GluR2/3 positive neurons was relatively small with largest at 250 μm^2 and the majority at around
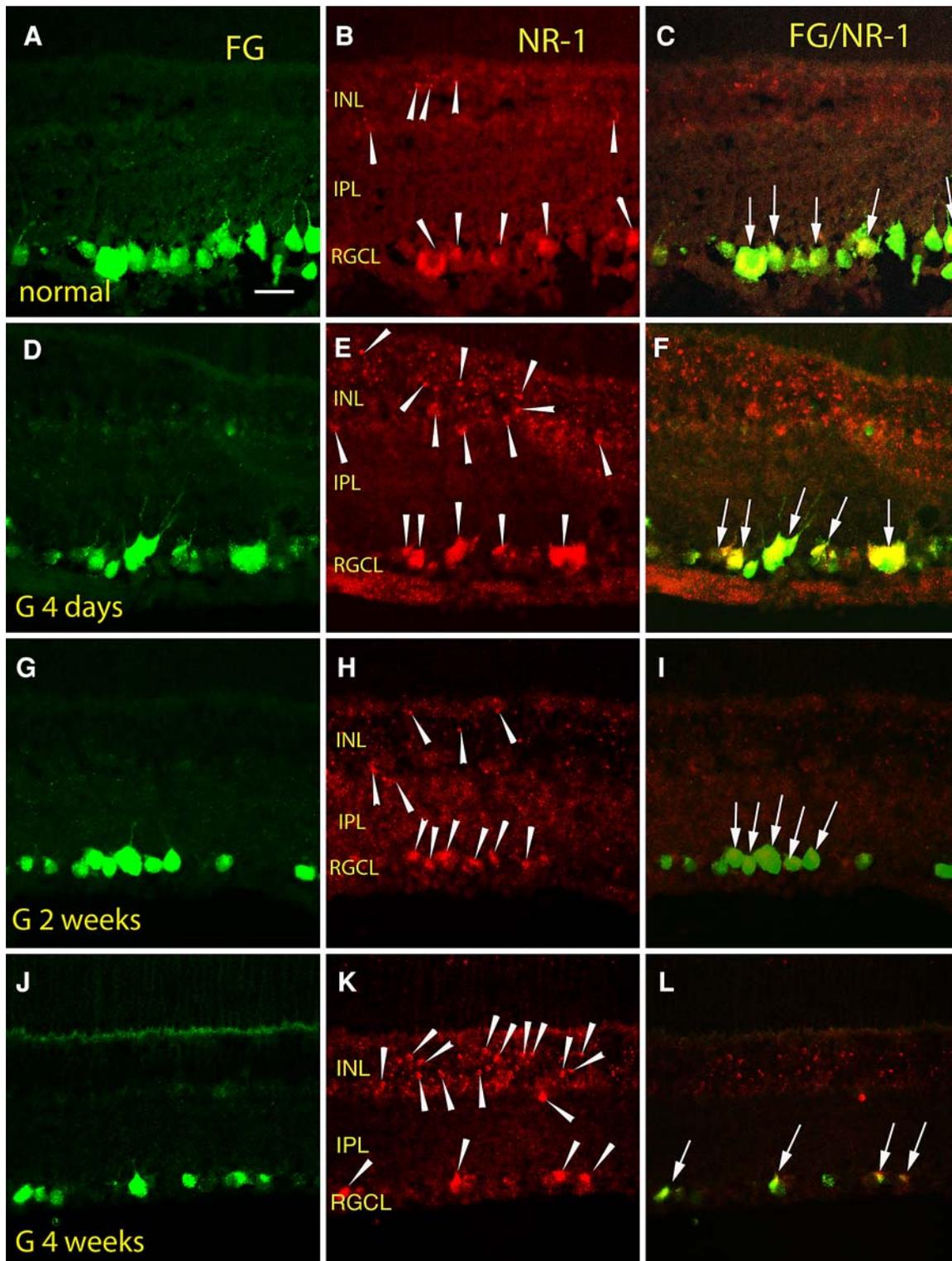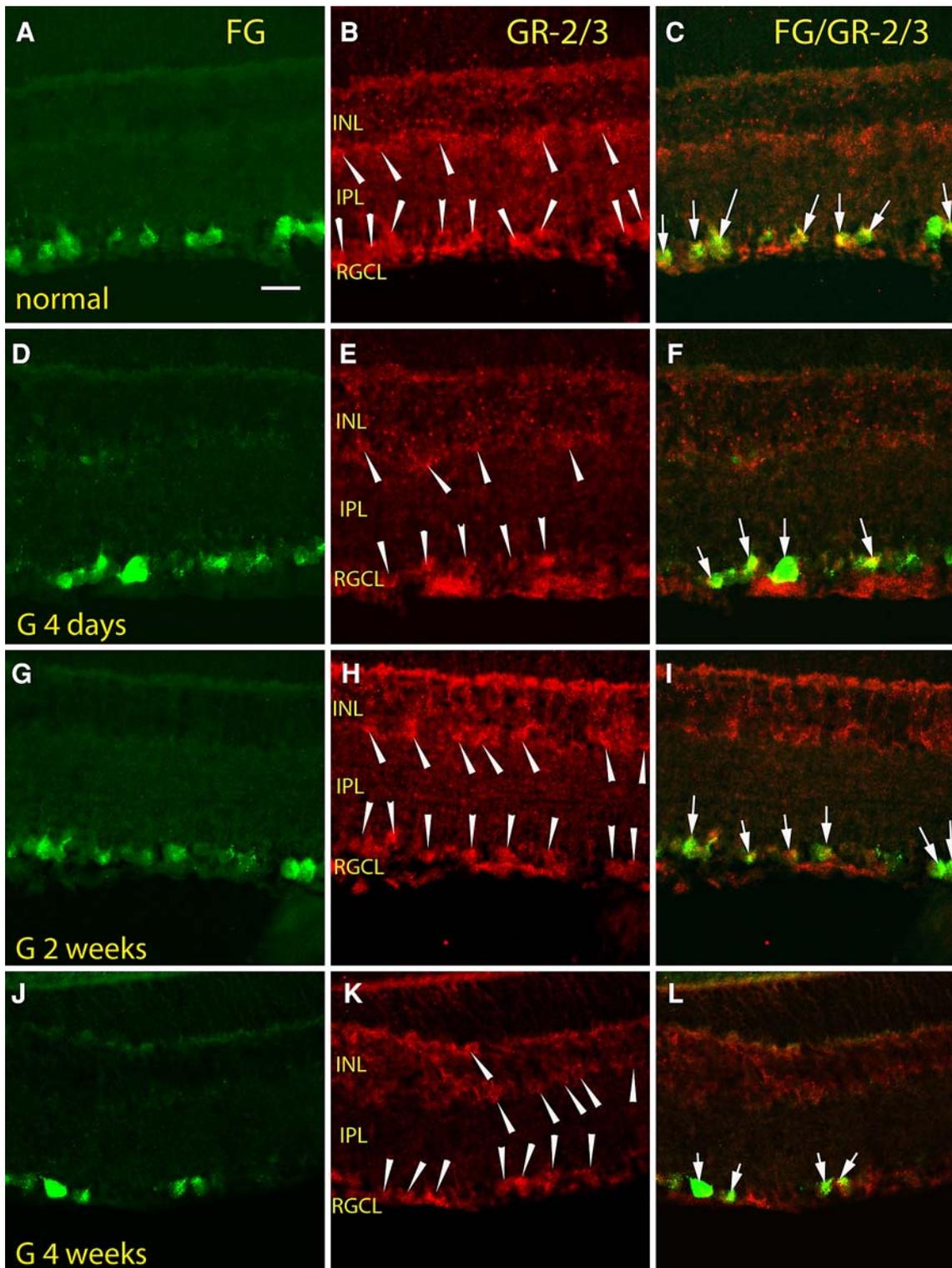
Fig. 3 Confocal micrographs of FG and NR1 positive RGCs in normal and glaucomatous retinas. FG labeled RGCs (**a**, **d**, **g**, **j**), immunohistochemical staining of NR1 (**b**, **e**, **h**, **k**, *arrowheads*) and merged (**c**, **f**, **i**, **l**, *arrows*) in normal control (**a**–**c**) and glaucomatous retinas (**d**–**l**). Some of the large RGCs were double labeled by FG and NR1 (*yellow* in **c**, **f**,) while reduced in 14 and 28 days glaucoma (**i**, **l**). *Scale bar* 20 μm. INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer. (Color figure online)

**Fig. 4** Confocal micrographs of FG and GluR2/3 positive RGCs in normal and glaucomatous retinas. FG labeled RGCs (**a**, **d**, **g**, **j**), immunohistochemical staining of GluR2/3 (**b**, **e**, **h**, **k**, *arrowheads*) and merged (**c**, **f**, **i**, **l**, *arrows*) in normal control (**a–c**) and glaucomatous retinas (**d–l**). Most of the double labeled RGCs by FG and GluR2/3 are medium in size (*yellow* in **c**, **f**, **i**, **l**). *Scale bar* 20 μm. INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer. (Color figure online)
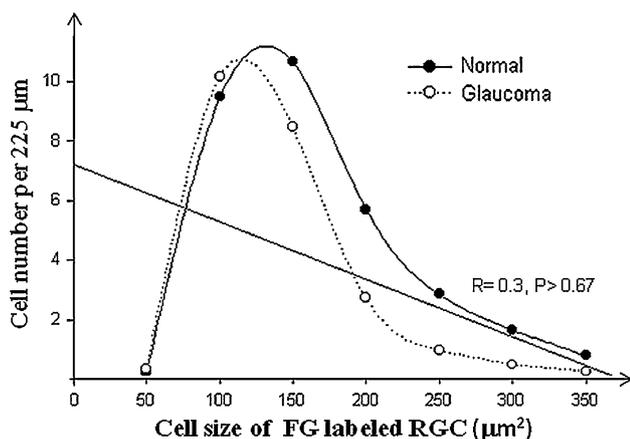
Fig. 5 Sectional area of FG labeled RGCs showing the number of RGCs was decreased in the glaucomatous retinas. It shows that the number of the small RGCs relatively increased while the large RGCs decreased severely but the correlation between the number of cell loss and cell size in the glaucomatous retinas was not significant ($R = 0.13$, $P > 0.5$)
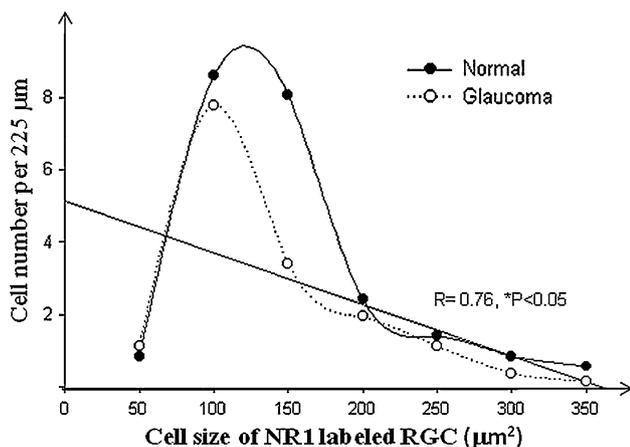

Fig. 7 Sectional areas of GluR2/3 positive RGCs showing that most of GluR2/3 positive RGCs were medium to small sized. It shows that the number of small to large GluR2/3 positive RGCs was slightly reduced in the glaucomatous retinas. There was no significant positive correlation between cell size and the number of cell loss ($R = 0.52$, $P > 0.05$)

Fig. 6 Sectional area of NR1 positive RGCs showing the number of RGCs was decreased in the glaucomatous retinas. Positive correlation between the cell size and the number of cell loss was shown by the *regression line* ($R = 0.76$, $P < 0.05$)

100 μm^2. And the loss of GluR2/3 positive RGCs was not significantly related with cell size ($R = 0.56$, $P > 0.36$, Fig. 7).

Discussion

Glutamate and its agonists are involved in the RGC loss in the pathology of glaucoma. The neurotoxic effect of glutamate is mediated by various subunits of glutamate receptors. The mRNA of the glutamate receptor subunits NR1, NR2A-C, KA2 and GluR6–7 are shown to be expressed in the RGCL of the rat retina using in situ hybridization (Brandstatter et al. 1994). Our study using
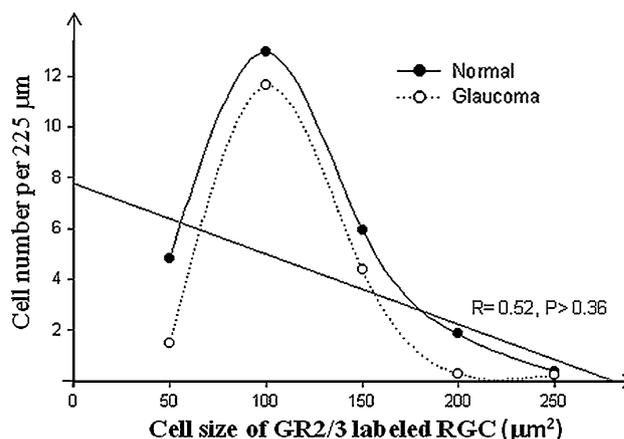
retrograde labeling and immunohistochemical methods identify simultaneously FG and NR1 or GluR2/3 in the same neuron has enabled us to study the distribution of NR1 or GluR2/3 in the surviving RGC in the normal and glaucomatous retinas.

In normal rat retina, about 80.4% RGC expressed NR1 and 85.4% RGC expressed GluR2/3 (Fig. 2a). Most of the NR1 positive RGCs were medium to large sized (Figs. 3, 6). While, the size of the RGCs labeled with GluR2/3 were of small to medium size (Figs. 4, 7). These findings suggest that there are selective expressions of glutamate receptor subunits in the RGCs and for a certain portion of RGCs, there were both NR1 and GluR2/3 expression.

NR1 is the major NMDA receptor subunit that is known to form the NMDA ion channel (Kutsuwada et al. 1992; Meguro et al. 1992; Monyer et al. 1992; Moriyoshi et al. 1991). A functional NMDA channel is suggested to be one of the key players to mediate glutamate neurotoxicity (Choi 1994). During the 28 days period of COH, the loss of NR1 positive RGC was about 26.7%. Compared with the 5.8% loss of GluR2/3 positive RGC and the overall loss of RGC at about 17%, the present results indicate that the subgroups of RGCs that are NR1-immunoreactive are more vulnerable to degeneration in the early phase of COH. However, the loss of NR1-positive RGCs did not start until around 4 days after the first laser and the expression of NR1 in RGCs was increased at the very first stage of onset of glaucoma (Fig. 2b). The upregulation of NR1 expression in the RGCs at early onset of ocular hypertension was consistent with the finding that the mRNA of NR1 was upregulated in the 1 week glaucomatous rat retina in the cauterization model (Kim et al. 2007). The excessive expression of NR1 in the RGCs may be involved in the

course of cellular degeneration of the RGCs in the early stage of glaucoma and leading to progressive RGC loss thereafter. This was proved in the monkeys chronic experimental glaucoma model with 21 to 416 weeks survival time, not only NR1 but also GluR2 positive neurons with neurofilament labeling were dramatically reduced over long observation period (Hof et al. 1998).

Compared with the upregulation of NR1 in RGCs during the first 4 days after the first laser, there was loss of GluR2/3 expression in RGCs. After that, the loss of GluR2/3 positive RGCs was stabilized at about 5.8% till the 28th day after the first laser. This may be related to the fact that GluR2 is considered as a "molecular switch" of the AMPA channel related to Ca^{2+} permeability in neurons (Bennett et al. 1996; Vandenberghe et al. 2000). With the expression of GluR2 subunit, the AMPA channels are less permeable to Ca^{2+}. The neurons with a low Ca^{2+} permeability, i.e., those displayed GluR2/3 immunoreactivity in this case, are likely to be less susceptible to glutamate-induced neurotoxicity. Internalization and/or downregulation of GluR2 were found in the early stage of ischemic or degenerative disease, that may related to excitotoxic neuronal death (Liu et al. 2006; Zhao et al. 2008). Similarly, in our study those RGCs showing down regulated expression of GluR2/3 at 4 days after the first laser might link to the switching on the NR1 overexpression in the same RGC and lead to dramatic loss of RGCs in the following time points.

The selective cell death, where by large RGCs were selectively lost in human and experimental glaucoma has been demonstrated previously by Glovinsky et al. (1991). Furthermore, glutamate or NMDA has been found to be more toxic to large RGCs both in tissue culture and in the intact rat eye while cells smaller than 10 μm diameter were relatively unaffected by glutamate or NMDA (Dreyer et al. 1994). Vickers et al.(1995) reported that large ganglion cells labeled with neurofilament specific antibodies were severely damaged in the glaucomatous eyes. Our study further confirmed these findings and demonstrated that compared with GluR2/3 positive RGCs, the NR1 positive RGCs were more vulnerable to increased intraocular pressure and the loss of NR1 positive RGCs were size dependent.

In summary, our data showed that NR1 positive large RGCs were the most vulnerable type under the stress of ocular hypertension. Early down regulation of GluR2/3 in the RGCs may trigger up regulation of the NR1 in the same RGC and involved in the early phase of ocular hypertension. However, the RGC death in glaucoma is a very complicated process involving the interaction of two or more receptors. Further works are needed to elucidate the mechanism of RGC death in glaucoma.

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