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Glaucoma-Induced Degeneration of Retinal Ganglion Cells Prevented by Hypoxic Preconditioning: A Model of Glaucoma Tolerance

Yanli Zhu,[1] Lihong Zhang,[1] Jimena F Schmidt,[2] and Jeffrey M Gidday[1,2,3]

[1]Department of Neurosurgery, [2]Ophthalmology and Visual Sciences, and [3]Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri, United States of America

Like all cells, neurons adapt to stress by transient alterations in phenotype, an epigenetic response that forms the basis for preconditioning against acute ischemic injury in the central nervous system. We recently showed that a modified repetitive hypoxic preconditioning (RHP) regimen significantly extends the window of ischemic tolerance to acute retinal ischemic injury from days to months. The present study was undertaken to determine if this uniquely protracted neuroprotective phenotype would also confer resistance to glaucomatous neurodegeneration. Retinal ganglion cell death at somatic and axonal levels was assessed after both 3 and 10 wks of sustained intraocular hypertension in an adult mouse model of inducible, open-angle glaucoma, with or without RHP before intraocular pressure elevation. Loss of brn3-positive ganglion cell soma after 3 wks of experimental glaucoma, along with increases in several apoptotic endpoints, were all significantly and robustly attenuated in mice subjected to RHP. Soma protection by RHP was also confirmed after 10 wks of intraocular hypertension by brn3 and SMI32 immunostaining. In addition, quantification of axon density in the postlaminar optic nerve documented robust preservation in RHP-treated mice, and neurofilament immunostaining also revealed preconditioning-induced improvements in axon integrity/survival in both retina and optic nerve after 10 wks of experimental glaucoma. This uniquely protracted period of phenotypic change, established in retinal ganglion cells by the activation of latent antiapoptotic, prosurvival mechanisms at both somatic and axonal levels, reflects a novel form of inducible neuronal plasticity that may provide innovative therapeutic targets for preventing and treating glaucoma and other neurodegenerative diseases.

INTRODUCTION

Glaucoma is the second leading cause of blindness worldwide, and its incidence is only expected to increase (1). Current pharmacological treatments designed to lower intraocular pressure (IOP) in primary open-angle glaucoma have shown some efficacy in delaying or preventing the onset of disease (2), but many eligible patients do not receive regular treatment or derive significant benefit from this approach, and many individuals develop glaucoma in the absence of significant intraocular hypertension. Thus, neuroprotective-based therapeutic approaches to prevent or slow the progressive loss of retinal ganglion cells (RGCs) that characterize this neuropathy have received considerable attention (3,4).

By leveraging the innate ability of cells to adapt to stressful conditions, a preconditioning-based approach to protecting RGCs from glaucomatous injury could provide therapeutic benefit. To date, however, epigenetic adaptations in the central nervous system (CNS) of experimental animals in response to a single preconditioning stimulus are very short-lasting, and "tolerance" has only been documented for acute injury. On the basis of our previous findings in adult mice that retinal ischemic tolerance could be extended from days to weeks by preconditioning in an intermittent, repeated manner before the insult (5,6), the present study was undertaken to test the hypothesis that inducing such a protracted window of augmented cellular viability might also prevent or ameliorate the progressive loss of RGC soma and axons that occurs in glaucoma.

MATERIALS AND METHODS

Experimental Groups

Two main groups of mice were studied: one group included mice sacrificed after 3 wks of sustained IOP elevation induced in one eye, used for quantify-

Address correspondence to *Jeffrey M. Gidday, Department of Neurological Surgery, Box 8057, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO, 63110. Phone: 314-286-2795; Fax: 314-286-2900; E-mail: gidday@wustl.edu.*

ing soma survival and apoptotic markers, along with quantification of axon density in the postlaminar optic nerve. A second group was sacrificed after 10 wks of intraocular hypertension, and soma survival, along with axon integrity in both the intraretinal nerve fiber layer and in the postlaminar optic nerve, was assessed. Randomly matched groups receiving repetitive hypoxic preconditioning (RHP) before IOP-elevating surgery were studied, as well as randomly matched controls without IOP-elevating surgery that included sham glaucoma mice (subjected to all anesthesia treatments and IOP measurements, but not episcleral vein ligation) and/or naïve mice; the contralateral eye served as the control for most endpoints examined.

Mouse Model of Experimental Glaucoma and RHP

All experimental methods and animal care procedures were conducted in accordance with NIH and ARVO guidelines for the care and use of laboratory animals and were approved by the Animal Studies Committee at Washington University. We used adult (10–12 wks old) male C57Bl/6J mice (20–30 g; Jackson Laboratory), randomized to 3- or 10-wk groups with respect to the duration of intraocular hypertension studied. Intraocular hypertension was induced by blocking the aqueous drainage of the eye secondary to repeated ligation of episcleral veins. In brief, mice were anesthetized with ketamine (87 mg/kg intraperitoneally [i.p.]) and xylazine (13 mg/kg i.p.), followed by topical application of 0.5% proparacaine hydrochloride. In one eye, the conjunctiva and Tenon's capsule were incised to expose the episcleral veins, three to five of which over 300 degrees of the limbus were ligated with 11-0 nylon suture (Alcon Surgical) under a dissecting microscope. Each vein was sutured at two points and then severed in between. Core body temperature was maintained at 37ºC throughout the procedure via a thermoregulated heating pad. Additional

ligations were performed at weekly intervals thereafter if IOP did not remain elevated. Antibiotic ointment was applied topically after each surgery; mice recovered in their home cages during the 3- or 10-wk period of elevated IOP. Randomly matched animals destined for each glaucoma cohort were exposed to RHP before the very first IOP-elevating surgery as follows: RHP involved exposing conscious mice for 2 h to a single period of systemic hypoxia (11% oxygen) three times per week for two consecutive weeks (5). The initial episcleral vein ligation surgery was then performed 3 d after the last hypoxic exposure.

Intraocular Pressure Measurements

IOP was measured before and at weekly intervals after the induction of intraocular hypertension by the TonoLab Rebound Tonometer system (Colonial Medical Supply, Franconia, NH, USA) following the manufacturer's recommendations and as described by others (7). See the supplemental materials for more details.

Clinical Symptomology Scoring

Several clinical symptoms common to human glaucoma (corneal limbal vessel dilation, anterior chamber cloudiness, corneal haze secondary to edema, hyphema and corneal bubbles) were scored weekly in a semiquantitative fashion to monitor and evaluate the response of the entire eye to the repeated episcleral vein ligation and the ongoing elevation in IOP and RHP. See the supplemental materials for details.

RGC Soma Survival Quantification

After 3 or 10 wks of elevated IOP, both untreated and RHP-treated animals were euthanized to quantify surviving RGC soma by quantification of brn3 immunopositive cells in peripheral regions of flat-mounted retinas, as described previously by us and others (8). See the supplemental materials for details.

RGC Axonal Survival Quantification

Axon integrity and survival was documented by two methods and in two loca-

tions. In the 3-wk glaucoma group, we quantified by light microscopy the density of morphologically normal axons in epon-embedded sections of postlaminar optic nerve cross-sections using p-phenylenediamine (9). In the 10-wk group, axon integrity and dropout was also assessed by confocal immunofluorescence microscopy within both the flat-mounted retina and the postlaminar optic nerve by SMI32 staining for the nonphosphorylated neurofilament heavy subunit, in conjunction with glial fibrillary acidic protein (GFAP) staining for assessing reactive astrogliosis. Some flat-mounted retinas were also immunostained with SMI34 to detect the cytoskeleton-associated, hyperphosphorylated neurofilament heavy subunit. Methodological details are included in the supplementary materials.

Immunoblotting and Immunohistochemistry

Retinas were collected under resting baseline conditions, immediately or 3 wks after RHP, or 3 wks after IOP elevation, to quantify retinal protein expression levels by immunoblotting (5). Paraffin-embedded cross-sections of retinas allowed us to immunohistochemically identify changes in expression and the cellular localization of the cleaved forms of both caspase-9 and -3 at 24 h, 1 wk, and 3 wks after intraocular hypertension. See the supplemental materials for details.

Statistics

Significant differences between measures from paired eyes in the same animal and from eyes in different animal groups were defined by nonparametric signed-rank and rank-sum tests, respectively. Nonparametric analysis of variance on ranks was used to identify quadrant-based differences within the retina and optic nerve. IOP comparisons among and between animals over the 3- and 10-wk periods of intraocular hypertension were defined by a repeated-measures linear model analysis using the mixed procedure of SAS (v9.2). $P < 0.05$ was accepted as significant.

RESULTS

IOP Changes in Our Model of Induced Glaucoma

Induction of experimental glaucoma by repeated episcleral vein ligation resulted in sustained, significant ($P < 0.05$) increases in IOP lasting at up to 10 wks, as long as newly appearing veins were ligated weekly. For both the 3- and 10-wk cohorts, baseline IOP did not differ significantly between the nonpreconditioned and preconditioned groups or between the experimental eyes and fellow eyes. Importantly, there was no overall difference in the IOP levels attained in the experimental eyes from untreated and RHP-treated groups, nor were there any statistically significant differences at any given time point between the elevated IOP level in each of these groups (Figures 1A, B), indicating that RHP was without effect on the IOP response to episcleral vein ligation.

Clinical Symptomology: IOP Elevation and Effects of RHP

Overall, we identified three patterns to the clinical scores. A correlation between the severity of symptoms and the intensity and duration of IOP elevation was expected, but the pattern of the correlation differed temporally, as did the severity of the clinical response, depending on whether the mice received prior RHP. These findings are detailed in Supplementary Table 1 and Supplementary Figures 1 and 2.

RHP Protects Retinal Ganglion Cell Soma in Glaucoma

We first assessed whether prior RHP would affect RGC soma death in response to sustained intraocular hypertension. Using regional analyses of retinal flat mounts immunostained for the RGC-specific antigen brn3 revealed that mice with prior RHP exhibited significantly higher numbers of surviving RGC soma compared with untreated mice in
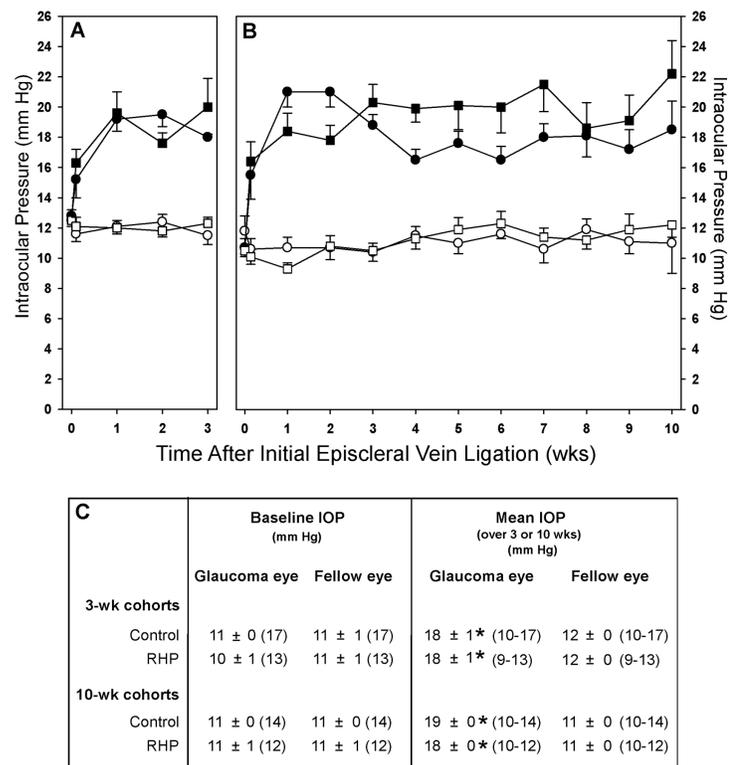

Figure 1. IOP changes in the different groups. Temporal changes in IOP in response to episcleral vein ligation in experimental eyes (filled symbols) and fellow eyes (open symbols) of mice from the 3-wk (A) and 10-wk (B) cohorts. IOP from untreated control mice (squares) and from mice treated before the initial episcleral vein ligation with RHP (circles) are shown. Although not indicated in the graphs for clarity, IOP levels in the experimental eyes were significantly greater at all time points than levels measured at corresponding times in their fellow eyes, and significantly greater than their respective baselines at all times. Moreover, there were no significant differences in the extent of IOP elevation in the glaucoma eyes from the untreated and RHP-treated groups, nor significant differences in IOP levels between these groups in their respective fellow eyes. Baseline IOP (shown at time = 0) also did not differ between eyes or groups. Baseline IOP values and mean IOP values over the 24-h–to–3-wk and 24-h–to–10-wk periods of study in the 3-wk and 10-wk cohorts, respectively, with the N for each group (in parentheses), are provided in tabular form (C), wherein *$P < 0.05$ versus baseline in the same eye.

both the 3-wk (Figure 2A) and 10-wk (Figures 2B, C) cohorts. Specifically, in the peripheral retina of nonpreconditioned mice (n = 7), $21 \pm 2\%$ ($P < 0.05$) of brn3-positive RGC soma were lost after 3 wks of intraocular hypertension relative to the fellow eye, but in RHP-treated mice (n = 6), this loss of cells was prevented by $91 \pm 9\%$ (that is, only $2 \pm 2\%$ of brn3-positive cells were lost) ($P < 0.05$). After 10 wks of experimental glaucoma, the magnitude of RGC soma loss had progressed to $30 \pm 4\%$ ($P < 0.05$ versus

fellow eyes) in the nonpreconditioned animals (n = 6). However, RHP-treated mice (n = 7) only lost $3 \pm 1\%$ of brn3-positive RGC soma in identical retinal regions, reflecting an $87 \pm 4\%$ improvement in survival ($P < 0.05$). RHP-induced protection was statistically equal across all quadrants, but was most robust in the temporal, superior and nasal quadrants (data not shown). Of note, the number of brn3-positive RGC soma in contralateral retinas did not differ between RHP-treated and untreated mice and were also
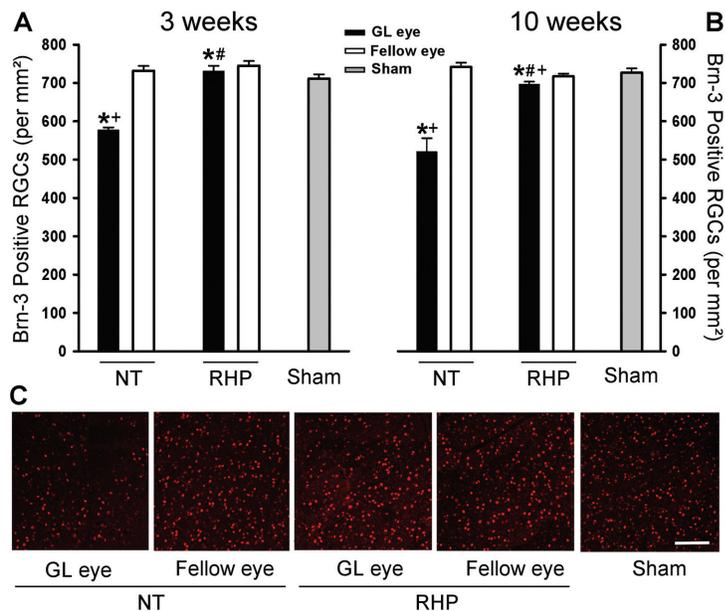
Figure 2. Total brn3-positive retinal ganglion cell soma density in peripheral retina as measured in retinal flat mounts from experimental (GL; black bars) and fellow eyes (open bars) obtained from untreated control mice (NT) and mice treated with RHP before intraocular hypertension. Results from both the 3-wk (A) and 10-wk (B, C) cohorts are shown, including those from sham mice (shaded bar) in both groups. Representative photomicrographs of brn3-labeled RGC soma (red) in peripheral retina of untreated and RHP-treated mice subjected to 10 wks of intraocular hypertension are also provided (C). *$P < 0.05$ versus fellow eye in each group; #$P < 0.05$ versus glaucomatous eye in NT group; +$P < 0.05$ versus respective sham. Scale bar in C = 50 μm.

indistinguishable from the number of brn3-positive RGC soma in the retinas of sham-glaucoma mice (n = 4; Figure 2B). Representative photomicrographs from untreated and RHP-treated groups of mice with 10 wks of experimental glaucoma are shown (Figure 2C). Overall, these findings indicate that RHP robustly protected against RGC soma loss, even after 10 wks of sustained IOP elevation.

Further evidence of RHP-induced RGC soma protection was revealed by immunostaining retinal flat mounts with SMI32, the dephosphorylated neurofilament heavy-chain label basally expressed by healthy RGCs (10–12), and SMI34, the cytoskeleton-associated, hyperphosphorylated neurofilament heavy subunit for which expression reflects cell injury and/or transport dysfunction (13). In particular, many SMI32-positive soma were evident across the peripheral retina of sham controls, but their density was

notably decreased, particularly in the superior and nasal quadrants, after 10 wks of glaucoma (n = 5), relative to soma densities observed in the corresponding retinal quadrants from controls, as well as relative to the densities of SMI32-positive soma noted in fellow nonglaucomatous retinas (Figure 3). In contrast, SMI32-positive soma density in the retinas of RHP-treated mice with glaucoma (n = 4) was indistinguishable from that in fellow eye and sham mice (see Figure 3). With respect to SMI34, RGC soma staining positively for this immunolabel were only found in nonpreconditioned glaucomatous retina (Supplementary Figure 3).

RHP Prevents RGC Apoptosis in Glaucoma

Given the evidence in both humans and animal glaucoma models that RGC soma are lost by apoptosis, we sought to

confirm (a) that the reductions in RGC soma densities we observed after sustained IOP elevation exhibited the cardinal features of apoptosis and (b) whether RHP protected RGC soma by an anti-apoptotic mechanism.

Immunohistochemistry for cleaved caspase-3 (Figure 4A) and cleaved caspase-9 (Figure 4B) revealed occasional, but prominent, increases in immunoreactivity for both in the cytoplasm of cells in the ganglion cell layer of mice with 3 wks of experimental glaucoma (n = 4–5), that in each case always colocalized with neuronal nuclei (NeuN)-positive cells, indicative of RGCs (Figure 4A). Neither cleaved caspase-3– nor cleaved caspase-9–positive cells could be found in fellow eyes or in nonglaucomatous controls (n = 3–4, data not shown). In examinations of glaucomatous retinas after 24 h or 1 wk of intraocular hypertension, no immunopositive cells for either enzyme could be found in the 24-h samples; and in the 1-wk samples, only rarely could we find a cleaved caspase-3 immunopositive cell (data not shown). By immunoblotting, we also measured a 70 ± 22% increase ($P < 0.05$), relative to normal controls (n = 6), in cleaved caspase-9 levels in the retinas of mice with 3 wks of elevated IOP (n = 6) (Figure 4C). In addition, we measured a 21 ± 10% decrease in the bcl-2/bax ratio (Figure 5) in retinas from the 3-wk intraocular hypertension cohort (n = 5), relative to that measured in retinas from matched control mice with normal IOP (n = 8).

Each of the aforementioned indices of apoptotic RGC death was significantly attenuated or reversed in animals with RHP. Specifically, we never found cleaved caspase-3– or cleaved caspase-9– immunopositive cells in the ganglion cell layer (or any other layer) of RHP-treated mice with 3 wks (or 1 wk or 24 h) of intraocular hypertension (n = 4; Figures 4A, B). In parallel with our immunohistochemistry findings, cleaved caspase-9 protein expression levels measured by immunoblot were no longer elevated ($P < 0.05$) in the glaucomatous retinas of RHP-treated mice (n = 6);

rather, the protein was expressed at a level that was $21 \pm 22\%$ below, but not significantly different from, normal IOP controls (Figure 4C). In addition, the retinas of RHP-treated mice with elevated IOP (n = 5) exhibited significantly higher ($P < 0.05$) bcl-2/bax ratios than glaucomatous retinas from mice without RHP (Figure 5). Moreover, measurements made immediately after the last RHP treatment (n = 5) and then again 3 wks later (n = 5), both before glaucoma, revealed a "priming effect" of RHP on the expression of these apoptosis-regulating proteins, with higher bcl-2/bax ratios at both time points (see Figure 5). Collectively, these findings indicate that RHP-induced increases in RGC soma survival occurred secondary to a prevention and/or reduction in apoptotic death of RGCs, even in the face of an equivalent magnitude and duration of intraocular hypertension.

RHP Protects Retinal Ganglion Cell Axons in Glaucoma

To determine if the soma survival-promoting effects of RHP were also evident in RGC axons, we quantified axonal density in postlaminar optic nerve cross-sections. Three weeks of sustained IOP elevation led to a $32 \pm 5\%$ total loss ($P < 0.05$) of RGC axons in these mice (n = 6) relative to the fellow optic nerve (Figure 6B). The glaucomatous optic nerve was characterized by a morphological pattern of disrupted and sometimes swollen axon bundles containing shrunken, degenerating and missing axons, typically concomitant with disruptions in myelin sheath integrity and a dark axoplasm (Figure 6A). In addition, more extensive and heterogeneously shaped astrocyte processes comprised a much greater proportion of nerves from glaucomatous eyes. Whereas axonal loss between quadrants was not significantly different, the inferior quadrant trended toward the least amount of loss relative to the other three quadrants (data not shown). In RHP-treated mice (n = 5), axonal morphology appeared similar to that in the fellow eye (axoplasms were clear,
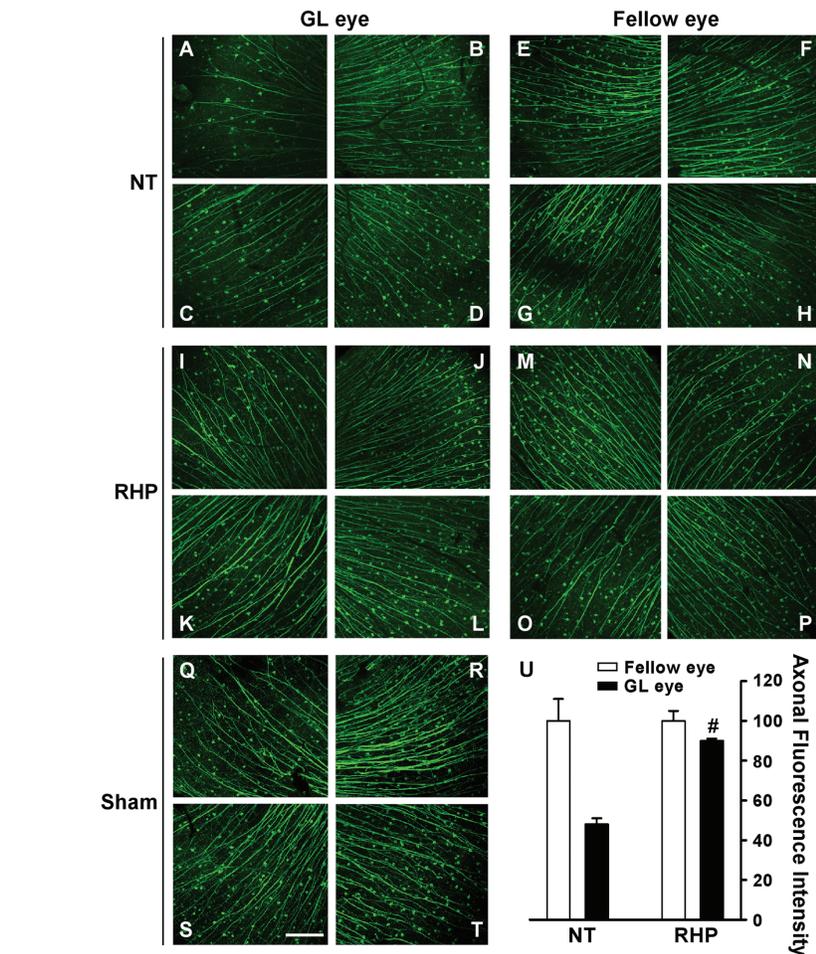

Figure 3. Confocal fluorescent immunohistochemistry for the nonphosphorylated neurofilament heavy-label SMI32 in retinal flat mounts from the experimental eye (GL) and respective fellow eye of representative untreated mice (NT) with 10 wks intraocular hypertension, mice receiving RHP before induction of 10 wks of intraocular hypertension, and sham controls. For each retina shown, superior regions are represented in panels A and B, E and F, I and J, M and N, and Q and R, and respective inferior regions are C and D, G and H, K and L, O and P, and S and T. Temporal regions are A and C, F and H, I and K, N and P, and Q and S, and nasal regions are B and D, E and G, J and L, M and O, and R and T. Scale bar is 200 μm. The histogram in panel U shows the quantified results for SMI32 fluorescence intensity across all quadrants, in all animals, normalized to fellow eyes in each group. [#]$P < 0.05$ versus GL retina in the NT group.

myelin sheaths were uninterrupted and intact axon fascicles were interspersed with normal-looking, stellate-shaped astrocytes), and $78 \pm 5\%$ of the total axonal loss was prevented (that is, only $8 \pm 2\%$ of all RGC axons were lost in mice with RHP [$P < 0.05$]; see Figure 6B). Again, no significant differences in the extent of RHP-induced protection was noted at the quadrant level, but protection in superior, inferior and temporal quadrants trended

most robust (data not shown). As with brn3-positive soma staining, RHP was without effect on axon density or any notable changes in optic nerve morphology (see Figure 6A), on the basis of comparisons between the fellow optic nerve of RHP-treated mice and the fellow optic nerve of nonpreconditioned mice.

The effect of RHP on RGC axonal viability in the postlaminar optic nerve was also investigated in mice with 10 wks of
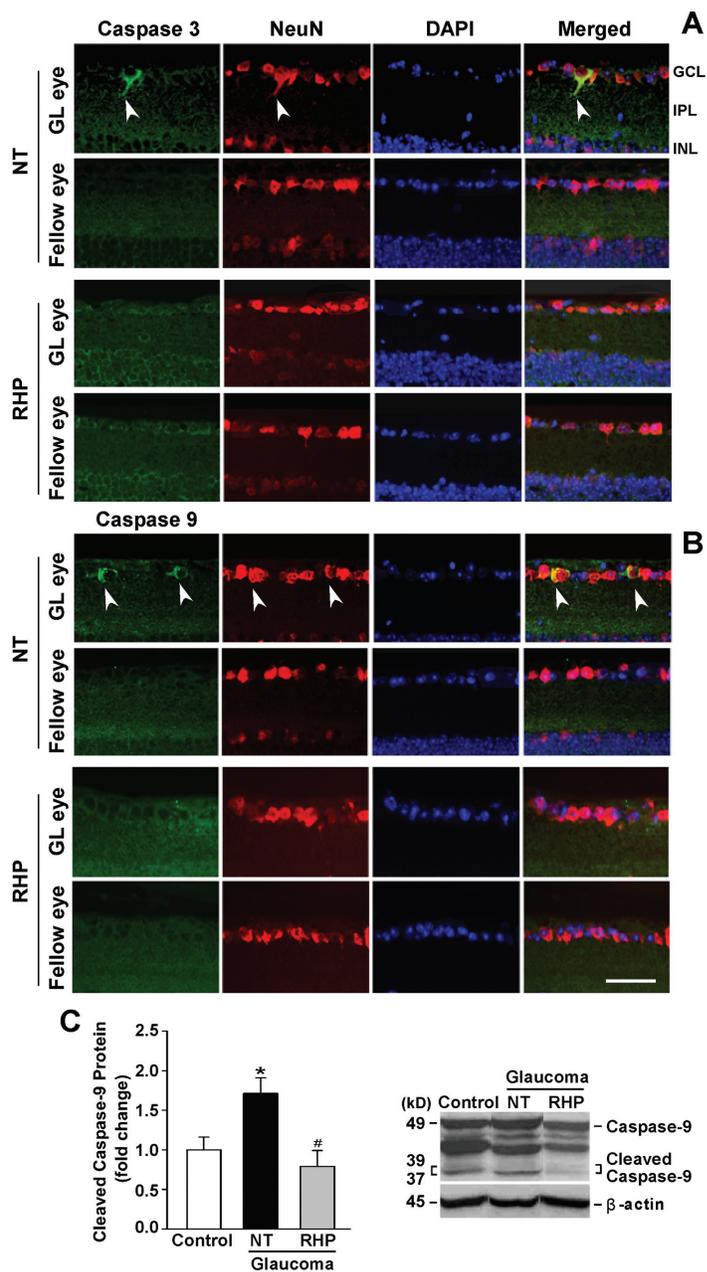
Figure 5. Retinal expression of bcl-2 and bax protein levels (shown as ratio) from mice sacrificed immediately or 3 wks after RHP and from mice with 3 wks of experimental glaucoma without (NT) or with RHP; all expression levels are normalized to those measured in untreated controls. Representative blots of the respective proteins, each with β-actin as a loading control, are shown below the histogram. *$P < 0.05$ versus the corresponding eye in the control group or NT group.

Figure 4. Immunohistochemical localization of the cleaved forms of caspase-3 (A) and caspase-9 (B) to retinal ganglion cells in retinas from untreated mice with 3 wks of intraocular hypertension. Caspases (green), NeuN (red), 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue) and merged images (yellow) are shown for each caspase, in the experimental glaucoma eye (GL) and in the fellow eye from untreated (NT) mice, and from mice with RHP before intraocular hypertension. Arrowheads in the GL eye of the NT group show immunopositive cleaved caspase-3 and -9 cells that colocalize with NeuN immunopositive cells. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar in each = 20 μm. (C) Western blot results for cleaved caspase-9 protein. On the left are quantified results in histogram form (normalized to sham controls) and on the right are representative blots from each experimental group, using β-actin as a loading control. *$P < 0.05$ versus the corresponding eye in the control group or NT group. #$P < 0.05$ versus the NT group.

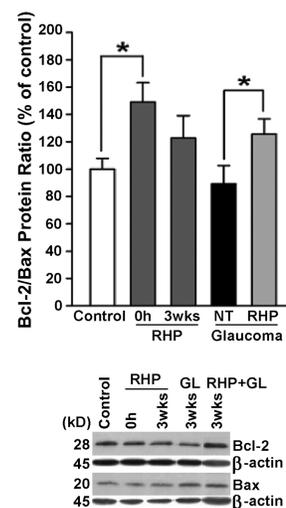glaucoma. As shown in representative photomicrographs (Figure 7), relative to the contralateral eye, clear reductions in the density of SMI32-positive axons in cross-sections of postlaminar optic nerve were evident in untreated mice (n = 4). Concomitantly, we consistently observed an increase in the intensity and the regional coverage of GFAP-positive astrocytes, the latter secondary to hypertrophy of their cell bodies and processes, and their filling in of the spaces created by axonal degeneration. Generally, more SMI32-positive axonal loss was evidenced in superior regions of the nerve relative to inferior. However, in mice receiving RHP (n = 4), a loss of SMI32-immunopositive axons was not evident in any region, and the nerve from the glaucomatous eye looked nearly identical to the fellow eye, and to shams, with respect to both axonal and glial density and morphology (see Figure 7). On the basis of the contention that reductions in

SMI32-positive axon density reflect overt axonal loss at the time of measurement (11), and taken collectively with our 3-wk axonal quantification data, our findings indicate that RHP exerted robust protective effects on RGC axons at the postlaminar level of the nerve, evident after both 3 and 10 wks of sustained intraocular hypertension.

We also examined RGC axon density and integrity more proximally, in the nerve fiber layer of the retina. There, we observed an obvious loss of SMI32-labeled axons running across the peripheral and mid-peripheral retina in all quadrants in untreated mice from the 10-wk glaucoma group (n = 5). The greatest loss was on the nasal side, and more prominently in the superior nasal quadrant, but there was still some loss on the temporal side as well (Figure 3). The integrity of axons still remaining also appeared somewhat jeopardized, with axonal and dendritic thinning, discontinuous segments and considerably less branching. When quantified across all quadrants, we found a 52 ± 3% loss (P = 0.063) of SMI32 + axons relative to fellow eyes (Figure 3U). However, in mice with prior RHP (n = 4), SMI32-positive axonal density and integrity in the nerve fiber layer appeared normal in all quadrants and qualitatively were indistinguishable from that in both contralateral retinas, as well as in retinas from sham glaucoma mice (see Figure 3). When quantified, axon integrity was improved significantly (P < 0.05) to within 10 ± 1% of that in their fellow eye (a degree of protection of 81%; see Figure 3U). Thus, the loss of proximal RGC axon segments within the nerve fiber layer in response to 10 wks of glaucoma was also robustly abrogated with RHP treatment.

DISCUSSION

Herein we show for the first time that repetitive preconditioning with sublethal hypoxia before disease onset can prevent the subsequent neurodegeneration characterizing glaucoma. The uniquely sustained adaptive response to RHP robustly abrogates the ongoing apoptotic
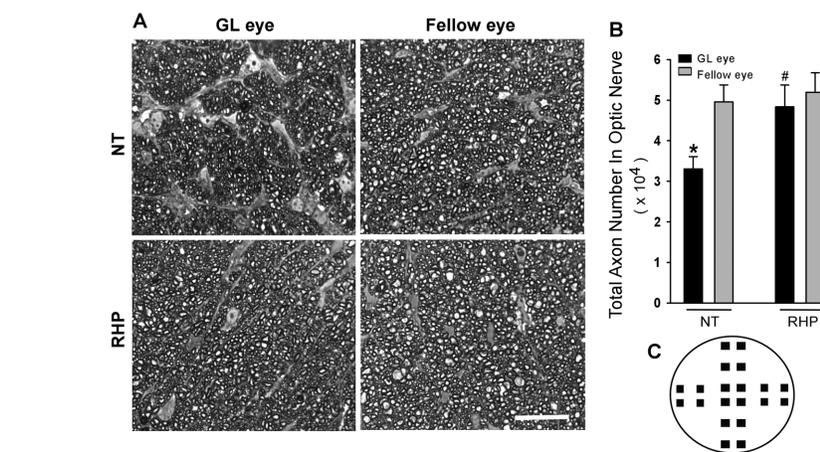

Figure 6. Representative photomicrographs (A) and summarized results (B) of axon densities in postlaminar optic nerve cross-sections from experimental eyes (GL) and fellow eyes from untreated mice with 3 wks of experimental glaucoma (NT) and from mice with RHP before 3 wks intraocular pressure elevation. *P < 0.05 versus fellow eye; #P < 0.05 versus GL eye in NT group. (C) Schematic of optic nerve cross section illustrating 20 individual regions over which axon density was determined. Scale bar in (A) = 20 μm.

death of RGC soma as well as axonal injury/loss. This novel demonstration of "glaucoma tolerance" implies the existence of intrinsic, cytoprotective regulatory systems in the CNS, the protracted activation of which can prevent or slow both the somatic and axonal degeneration associated with this disease. In turn, these findings advocate for an expansion of the traditional definitions of preconditioning and tolerance in the CNS to incorporate this novel form of induced neuroplasticity, and suggest the exciting possibility of achieving similarly protracted periods of protection against chronic cellular injury in other tissues.

In glaucoma, RGC axons die by mechanisms distinct from those governing the demise of the soma (11,14–20). Thus, we measured RGC viability at both somatic and axonal levels in the present study, not to elucidate primary and secondary injury events per se, but to demonstrate the potential pan-cellular, multifactorial protective effects of RHP. With respect to RGC soma, RHP robustly attenuated the 20–30% progressive loss of RGCs we documented by brn3 immunolabeling during the initial 3–10 wks of intraocular hypertension. The brn3-positive cell bodies we quantified comprise a specific, but

relatively large, subpopulation of RGCs that express this RGC-specific gene product (8); displaced amacrine cells are not identified with this immunolabel. Although the relative susceptibility to glaucomatous injury of RGCs carrying the brn3 gene compared with other RGC subpopulations is unclear, the magnitude of RGC soma loss in our model was similar to that independently measured in other inducible mouse glaucoma models (21–24). RHP-induced soma protection was also confirmed by immunolabeling for the nonphosphorylated neurofilament heavy-chain marker SMI32. The nuclear condensation of the label, truncation of positively stained dendrites and somatic shrinking we witnessed in nonpreconditioned glaucomatous retinas, described previously for the DBA/2J model (11), defines an RGC soma phenotype that was not observed in RHP-treated mice. Finally, our finding of hyperphosphorylated SMI34-positive RGC soma, axons and dendrites only in the glaucomatous retinas of nonpreconditioned mice is consonant with similar soma labeling in mouse RGCs disconnected from their distal axons in the DBA/2J model (19) and in rat RGCs after optic nerve crush (10); the shifting of
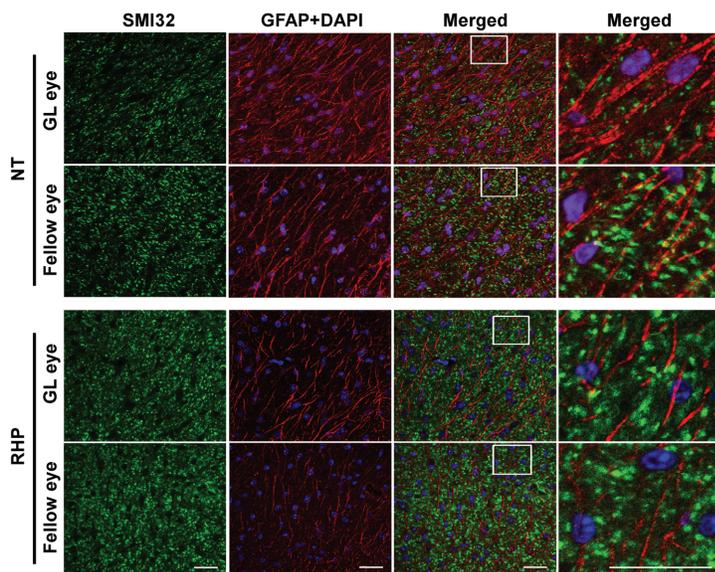
Figure 7. Confocal fluorescence immunohistochemistry in representative postlaminar optic nerve cross-sections from eyes with 10 wks experimental glaucoma (GL), and fellow eyes, in untreated mice (NT; upper two rows) and mice with RHP (lower two rows). Shown left to right, by columns, are SMI32 (green), GFAP (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) and two merged photo sets, with the right-most being a magnified view of the box shown in each respective photo to its left. Scale bars shown = 20 μm.

neurofilament phosphorylation from axon to soma is common to many neurological disorders and may be a harbinger for the eventual demise of the cell (13). Taken together, our findings support the concept that innate responses can be induced in RGCs by preconditioning that promote the survival of the soma in the face of neurodegeneration-inducing glaucoma.

Histological, biochemical, molecular and genetic evidence, in animal models (14,15,21,25–27), monkeys (28) and humans (29), collectively support the contention that RGC soma loss in glaucoma occurs by apoptosis. Our documentation of expected changes in several apoptotic endpoints in the current study are consonant with previous findings in these different models with respect to the altered expression of cleaved caspase-9 (30), cleaved caspase-3 (21,26) and bcl-2/bax (21,26,31,32). That changes in these aforementioned apoptotic endpoints were largely abrogated in RHP-treated mice indicates that RHP clearly established a robust antiapoptotic phenotype for RGC

soma. In fact, at least for bcl-2 and bax, we confirmed that RHP appears to "prime the pump" for such an effect, given the changes we observed after RHP in mice without subsequent IOP elevation. Whether RHP promotes such a phenotype secondary to transcriptional and/or posttranslational regulation of these and other proteins is not yet known. Overall, our results indicate that, mechanistically, when RHP precedes the period of intraocular hypertension, the somatic expression of several hallmark pro- and antiapoptotic proteins is altered in a sustained fashion in the retina such that the apoptotic demise of RGC soma is dramatically reduced.

With respect to RGC axons, our findings demonstrate that RHP treatment robustly protected against glaucomatous axonal loss, both distally in the postlaminar optic nerve and more proximally in the retinal nerve fiber layer. This result is a critical finding, given the accumulating evidence indicating that, in glaucoma and other neurodegenerative diseases, axonal injury is a fundamental, and per-

haps primary, event that ultimately leads to the apoptotic death of the soma (11,18,19). Although the extent and pattern of IOP elevation varies between models, and temporal correlations are not exact, the 32% axonal loss in the postlaminar optic nerve we quantified after 3 wks of IOP elevation was similar to the magnitude of loss (20–50%) observed after 2–4 wks in other inducible mouse glaucoma models (24,33). We also quantified robust preservation of proximal RGC axon integrity within the retina in RHP-treated mice. Because of their role in regulating and, to a minor extent, participating in axonal transport secondary to microtubule contacts, changes in phosphorylation status of the different neurofilament subunits can serve as surrogate markers for axonal injury and degeneration (34). Because SMI32 recognizes a nonphosphorylated epitope on the neurofilament heavy subunit that comprises the axonal cytoskeleton in healthy RGCs (11,12,35), expression changes are thought to reflect axonal dysfunction or degeneration and not a reduction in neurofilament transport; however, whether reductions in SMI32 labeling truly reflect axonal loss remains to be demonstrated conclusively. The greater axonal loss in superior regions of the postlaminar optic nerve that we observed at 10 wks is consistent with the regional difference reported for adult mice 12 wks after IOP elevation by limbal laser photocoagulation (36). Similar changes also occur in progressive fashion in the DBA/2J model with advancing disease (11,18,19).

Collectively, our quantitative and qualitative assessments of axonal integrity, at two locations and at two distinct time points, make it clear that the preservation of RGC axons was promoted by prior RHP treatment despite the protracted period of intraocular hypertension that causes significant axonal loss in untreated mice. Although this protection may, in part, be secondary to RHP-induced changes in astrocytes, oligodendrocytes, non-RGC neurons and/or other cells, the endogenous, intra-axonal

protective mechanisms that are uniquely activated by RHP to prevent or slow this axonopathy will be important to elucidate not only for this disease, but for brain and spinal cord injury and other white matter neuropathies. In glaucoma, this step may involve reductions in the extent of distal and proximal axonal transport deficits; inhibition of calcium influx, modulations in amyloid precursor protein (APP), caspase-6, nicotinamide mononucleotide adenylyltransferase (Nmnat), Jun NH$_2$-terminal kinase (JNK) or other putative mediators of the Wallerian degeneration-resistant phenotype; and perhaps even synaptic and other physiological adaptations at the level of the superior colliculus (17,20). The lack of axonal protection in DBA/2J mice deficient in bax (14) would suggest the axon survival-promoting effects of RHP are bax-independent and largely distinct from the antiapoptotic-based protection that we showed RHP affords to RGC soma.

Many studies support the contention that astrocytes (particularly those in the optic nerve head) contribute importantly to glaucoma pathology (37). Moreover, glial cell activation, as evidenced by the prototypical upregulation of GFAP, is thought to be a hallmark of CNS injury. However, this prevailing wisdom is being countered by the notion that, in glaucoma, reactive astrogliosis and other functional/morphological responses of astrocytes may actually be protective for nearby axons and soma depending on the spatio-temporal context of the response to elevated IOP (38). The astrocyte hypertrophy and enhancement in GFAP immunostaining intensity that we observed in the postlaminar optic nerve are well-established, relatively early responses in other inducible (27) and genetic (11,18,39) glaucoma models and may be a progressive response to fill spaces vacated by degenerating axons (11,18,35,40). That these glial changes did not occur in mice with prior RHP may reflect an astrocyte-specific adaptive response that contributes to the preservation of neighboring axons, or it may simply be that this secondary response to axonal loss was never initiated because RGC axonal dysfunction/loss was minimized by preconditioning.

Somatic and axonal protection of RGCs by RHP was demonstrated herein using an inducible mouse model of primary open-angle glaucoma in which a sustained, moderate elevation in IOP was achieved by weekly ligation of patent episcleral veins. Another group performed episcleral vein ligation in mice a single time and also obtained similar levels of RGC loss after a couple of weeks (23); however, as also observed in rats (25, 27), elevations in IOP were not as consistently maintained in these single ligation models. Laser photocoagulation of limbal and episcleral veins (21,22,33) and injection of microbeads (24) represent other ways of inducing transient IOP elevations in mice. Whereas reviews of the pros and cons of these rodent models will likely continue (16), we would predict that the robust RGC survival-promoting effects of RHP that we documented in our model will ultimately be demonstrable in other inducible models of glaucoma and perhaps other neurodegenerative diseases, given that preconditioning-induced ischemic tolerance appears to reflect fundamental, evolutionarily conserved, adaptive mechanisms on the part of all the cells in the body (41).

CONCLUSION

In conclusion, by repetitively preconditioning mice with intermittent exposures to noninjurious hypoxia (before disease initiation), RGC injury/death at both somatic and axonal levels was robustly attenuated after sustained periods of elevated IOP. This demonstration is the first to show promotion of a protracted period of endogenous neurovascular plasticity for preventing the loss of vulnerable RGCs in an experimental model of neurodegeneration. The ability to induce such a sustained, cell death–resistant phenotype may be therapeutically advantageous, not only for protecting the vision of glaucoma patients, but for saving neurons in other neurodegenerative diseases as well.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Quigley HA, Broman AT. (2006) The number of people with glaucoma worldwide in 2010 and 2020. *Br. J. Ophthalmol.* 90:262–7.
2. Peeters A, *et al.* (2010) Quantifying the effect of intraocular pressure reduction on the occurrence of glaucoma. *Acta Ophthalmol.* 88:5–11.
3. Cheung W, Guo L, Cordeiro MF. (2008) Neuroprotection in glaucoma: drug-based approaches. *Optom. Vis. Sci.* 85:406–16.
4. Levin LA, Peeples P. (2008) History of neuroprotection and rationale as a therapy for glaucoma. *Am. J. Manag. Care.* 14:11–4.
5. Zhu Y, Zhang Y, Ojwang BA, Brantley MA Jr, Gidday JM. (2007) Long-term tolerance to retinal ischemia by repetitive hypoxic preconditioning: role of HIF-1alpha and heme oxygenase-1. *Invest. Ophthalmol. Vis. Sci.* 48:1735–1743.
6. Zhu Y, Zhang L, Gidday JM. (2008) Deferroxam-

ine preconditioning promotes long-lasting retinal ischemic tolerance. *J. Ocul. Pharmacol. Ther.* 24:527–35.

7. Wang WH, Millar JC, Pang IH, Wax MB, Clark AF. (2005) Noninvasive measurement of rodent intraocular pressure with a rebound tonometer. *Invest. Ophthalmol. Vis. Sci.* 46:4617–21.

8. Leahy KM, *et al.* (2004) Quantitative ex vivo detection of rodent retinal ganglion cells by immunolabeling Brn-3b. *Exp. Eye Res.* 79:131–40.

9. Zhong L, *et al.* (2007) Erythropoietin promotes survival of retinal ganglion cells in DBA/2J glaucoma mice. *Invest. Ophthalmol. Vis. Sci.* 48:1212–8.

10. Dieterich DC, *et al.* (2002) Partial regeneration and long-term survival of rat retinal ganglion cells after optic nerve crush is accompanied by altered expression, phosphorylation and distribution of cytoskeletal proteins. *Eur J. Neurosci.* 15:1433–43.

11. Jakobs TC, Libby RT, Ben Y, John SWM, Masland RH. (2005) Retinal ganglion cell degeneration is topological but not cell type specific in DBA/2J mice. *J. Cell Biol.* 171:313–25.

12. Raymond ID, Pool AL, Vila A, Brecha NC. (2009) A Thy1-CFP DBA/2J mouse line with cyan fluorescent protein expression in retinal ganglion cells. *Vis. Neurosci.* 26:453–65.

13. Drager UC, Hofbauer A. (1984) Antibodies to heavy neurofilament subunit detect a subpopulation of damaged ganglion cells in retina. *Nature.* 309:624–6.

14. Libby RT, *et al.* (2005) Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. *PLoS Genet.* 1:17–26.

15. Nickells RW, Semaan SJ, Schlamp CL. (2008) Involvement of the Bcl2 gene family in the signaling and control of retinal ganglion cell death. *Prog. Brain Res.* 173:423–35.

16. McKinnon SJ, Schlamp CL, Nickells RW. (2009) Mouse models of retinal ganglion cell death and glaucoma. *Exp. Eye Res.* 88:816–24.

17. Crish SD, Calkins DJ. (2011) Neurodegeneration in glaucoma: progression and calcium-dependent intracellular mechanisms. *Neuroscience.* 176:1–11.

18. Howell GR, *et al.* (2007) Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. *J. Cell Biol.* 179:1523–37.

19. Soto I, *et al.* (2008) Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. *J. Neurosci.* 28:548–61.

20. Vohra BPS, *et al.* (2010) Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenylyltransferase 1-sensitive pathway. *J. Neurosci.* 30:13729–38.

21. Ji J, *et al.* (2005) Effects of elevated intraocular pressure on mouse retinal ganglion cells. *Vision Res.* 45:169–79.

22. Nakazawa T, *et al.* (2006) Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. *J. Neurosci.* 26:12633–41.

23. Ruiz-Ederra J, Verkman AS. (2006) Mouse model of sustained elevation in intraocular pressure produced by episcleral vein occlusion. *Exp. Eye Res.* 82:879–84.

24. Sappington RM, Carlson BJ, Crish SD, Calkins DJ. (2010) The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest. Ophthalmol. Vis. Sci.* 51:207–16.

25. Mittag TW, *et al.* (2000) Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model. *Invest. Ophthalmol. Vis. Sci.* 41:3451–9.

26. McKinnon SJ, *et al.* (2002) Caspase activation and amyloid precursor protein cleavage in rat ocular hypertension. *Invest. Ophthalmol. Vis. Sci.* 43:1077–87.

27. Yu S, Tanabe T, Yoshimura N. (2006) A rat model of glaucoma induced by episcleral vein ligation. *Exp. Eye Res.* 83:758–70.

28. Quigley HA, *et al.* (1995) Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest. Ophthalmol. Vis. Sci.* 36:774–86.

29. Kerrigan LA, Zack DJ, Quigley HA, Smith SD, Pease ME. (1997) TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch. Ophthalmol.* 115:1031–5.

30. Huang W, *et al.* (2005) Transcriptional upregulation and activation of initiating caspases in experimental glaucoma. *Am. J. Pathol.* 167:673–81.

31. Chierzi S, *et al.* (1998) Protection of retinal ganglion cells and preservation of function after optic nerve lesion in bcl-2 transgenic mice. *Vision Res.* 38:1537–43.

32. Lingor P, Koeberle P, Kugler S, Bahr M. (2005) Down-regulation of apoptosis mediators by RNAi inhibits axotomy-induced retinal ganglion cell death in vivo. *Brain.* 128:550–8.

33. Mabuchi F, Aihara M, Mackey MR, Lindsey JD, Weinreb RN. (2003) Optic nerve damage in experimental mouse ocular hypertension. *Invest. Ophthalmol. Vis. Sci.* 44:4321–30.

34. Balaratnasingam C, Morgan WH, Bass L, Cringle SJ, Yu D-Y. (2008) Time-dependent effects of elevated intraocular pressure on optic nerve head axonal transport and cytoskeleton proteins. *Invest. Ophthalmol. Vis. Sci.* 49:986–99.

35. Sun D, Lye-Barthel M, Masland RH, Jakobs TC. (2009) The morphology and spatial arrangement of astrocytes in the optic nerve head of the mouse. *J. Comp. Neurol.* 516:1–19.

36. Mabuchi F, Aihara M, Mackey MR, Lindsey JD, Weinreb RN. (2004) Regional optic nerve damage in experimental mouse glaucoma. *Invest. Ophthalmol. Vis. Sci.* 45:4352–8.

37. Hernandez MR, Miao H, Lukas T. (2008) Astrocytes in glaucomatous optic neuropathy. *Prog. Brain Res.* 173:353–73.

38. Johnson EC, Morrison JC. (2009) Friend or foe? Resolving the impact of glial responses in glaucoma. *J. Glaucoma.* 18:341–53.

39. Inman DM, Horner PJ. (2007) Reactive nonproliferative gliosis predominates in a chronic mouse model of glaucoma. *Glia.* 55:942–53.

40. Son JL, *et al.* (2010) Glaucomatous optic nerve injury involves early astrocyte reactivity and late oligodendrocyte loss. *Glia.* 58:780–9.

41. Gidday JM. (2006) Cerebral preconditioning and ischaemic tolerance. *Nat. Rev. Neurosci.* 7:437–48.