

Enhanced Retinal Ganglion Cell Survival in Glaucoma by Hypoxic Postconditioning After Disease Onset

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Abstract The neuroprotective efficacy of adaptive epigenetics, wherein beneficial gene expression changes are induced by nonharmful “conditioning” stimuli, is now well established in several acute, preclinical central nervous system injury models. Recently, in a mouse model of glaucoma, we demonstrated retinal ganglion cell (RGC) protection by repetitively “preconditioning” with hypoxia prior to disease onset, indicating an epigenetic approach may also yield benefits in chronic neurodegenerative disease. Herein, we determined whether presenting the repetitive hypoxic stimulus after disease initiation [repetitive hypoxic “postconditioning” (RH-Post)] could afford similar functional and morphologic protection against glaucomatous RGC injury. Chronic elevations in intraocular pressure (IOP) were induced unilaterally in adult male C57BL/6 mice by episcleral vein ligation. Mice were randomized to an RH-Post [1 h of systemic hypoxia (11 % oxygen) every other day, starting 4 days after IOP elevation] or an untreated control group. After 3 weeks of experimental glaucoma, the 21–27 % reduction and 5–25 % prolongation in flash visual-evoked potential amplitudes and latencies, respectively, and the 30 % impairment in visual acuity were robustly improved in RH-Post-treated mice, as was the 17 % loss in

RGC soma number and 20 % reduction in axon integrity. These protective effects were observed without RH-Post affecting IOP. The present findings demonstrate that functional and morphologic protection of RGCs can be realized by stimulating epigenetic responses during the early stages of disease, and thus constitute a new conceptual approach to glaucoma therapeutics.

Key Words Glaucoma · neuroprotection · epigenetics · retina · optic nerve

Introduction

Glaucoma includes a family of optic neuropathies that collectively represent the second leading cause of blindness in the world [1]. Neuroprotection-based therapies for protecting retinal ganglion cells (RGCs) from the damage induced by progressive and sustained increases in intraocular pressure (IOP) have yet to be successful in the limited number of clinical trials to date, leaving surgery or IOP-lowering drugs as the clinical mainstays for treating patients with this disease. Because RGC injury pathways in glaucoma are multifactorial and involve both soma- and axon-dependent mechanisms, it is unlikely that a monotherapy-based treatment strategy will ever prove viable.

Accumulating evidence indicates that a mechanistically pleiotropic approach to neuroprotection in acute central nervous system (CNS) injury can be afforded by an adaptive epigenetics-based therapy, wherein beneficial changes in gene expression are induced by a mild “conditioning” stressor [2–4]. In the setting of retina ischemia, we, and others, have shown that a brief, noninjurious hypoxic or ischemic

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preconditioning stimulus provides a short window of protection for RGCs and other retinal cells [5–7]. That the duration of this “therapeutic window” could be extended from days to weeks by repeatedly exposing mice to systemic hypoxia prior to ischemia [8] led us to hypothesize that this treatment approach might also be protective against the slow loss of RGCs that characterizes glaucoma. Indeed, we recently documented robustly enhanced survival of RGC soma and axons in an inducible mouse model of open-angle glaucoma by repetitive hypoxic preconditioning (RH-Pre) [9].

Translational speaking, the ability to activate such an innate protective response in patients already presenting with the early stages of glaucoma, and to significantly reduce the progression of RGC injury (and consequent vision loss), would represent a unique, clinically attractive therapeutic strategy for this disease. Thus, the present study was undertaken to determine whether “postconditioning” with repetitive hypoxia (RH-Post), initiated after IOP elevation, could still afford significant RGC protection in experimental glaucoma.

Methods

Animals

All experimental methods and animal care procedures employed were approved by the Animal Studies Committee at Washington University, and performed in accordance with National Institutes of Health and Association for Research in Vision and Ophthalmology guidelines for the care and use of laboratory animals.

Mice were randomized into either an untreated glaucoma group or a glaucoma group with RH-Post. This involved a total of 7, 1-h exposures of the animals to systemic hypoxia (11 % oxygen) over the 3 weeks following IOP elevation; the initial RH-Post stimulus of the 7 exposures was administered starting 4 days after episcleral vein ligation (Fig. 1).

Experimental Glaucoma Model

As we described previously [9–11], a sustained, 3-week period of elevated IOP was achieved by repeated ligation of the episcleral veins of one eye; the fellow eye of each mouse served as a nonglaucomatous control. In brief, after ketamine/xylazine-anesthetized mice received topical application of 0.5 % proparacaine hydrochloride, the conjunctiva and Tenon’s capsule were incised to expose the episcleral veins, 3–4 of which over $>300^\circ$ of the limbus were severed secondary to doubly ligating them with 11-0 nylon suture (Alcon Surgical, Fort Worth, TX, USA). Throughout the surgical procedure, core body temperature

was maintained at 37 °C using a thermoregulated heating pad. Mice were administered topical antibiotic and then allowed to recover in their home cages. Additional ligations of ≥ 1 presumably patent episcleral veins were performed at 24 h, and at 1 or 2 weeks following the initial surgery, if IOP was not elevated at 24 h or did not remain elevated at weekly measurement intervals, or if newly formed episcleral veins became evident. Of note, in this study and in our previous studies employing this same model [9–11], very few mice required additional ligation surgery at 24 h or 2 weeks after the initial ligation; rather, in most mice, new veins that became apparent by 1 week were ligated, even if IOP had not fallen below levels measured at 24 h. As described previously by us and others [9–12], IOP determinations were made between 10.00 am and 2.00 pm in both eyes prior to, and at weekly intervals following, IOP elevation using the TonoLab Rebound Tonometer system (Colonial Medical Supply, Franconia, NH, USA).

Elevated IOP was also induced in several other cohorts of animals to examine the possibility that inner retinal ischemia may result from our episcleral vein ligation procedure. Distinct ischemic injury phenotypes were assessed in central and peripheral retinal cross-sections obtained 24 h, 1 week, and 3 weeks after IOP elevation, including determinations of retinal cell layer and plexiform layer thinning, cleaved caspase-9 and cleaved caspase-3 protein expression, and the presence of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells.

Structural Analyses of Potential Ischemic Injury

Five-micron-thick cross-sections were obtained from paraffin-embedded retinas of mice in which IOP was elevated continuously for either 24 h, 1 week, or 3 weeks. Adjacent sections were stained by hematoxylin and eosin, prepared for TUNEL staining by a commercially available kit (ApopTag Fluorescein In situ Apoptosis Detection Kit, EMD Millipore, Billerica, MA, USA) according to manufacturer’s directions, or immunostained for cleaved caspase-3 and cleaved caspase-9 expression levels. For the latter, the primary antibodies used were from Cell Signaling [Danvers, MA, USA; #9664 and #9509 (for cleaved caspase-3 and cleaved caspase-9, respectively), both 1:100 for 2 h at room temperature], with AlexaFluor 488 (A-21206; lot #913921, 1:400 for 1 h at room temperature; Invitrogen, Carlsbad, CA, USA) as the secondary antibody. We searched for caspase 3/9-immunopositive and TUNEL-positive cells across the entire (central and peripheral) retinae.

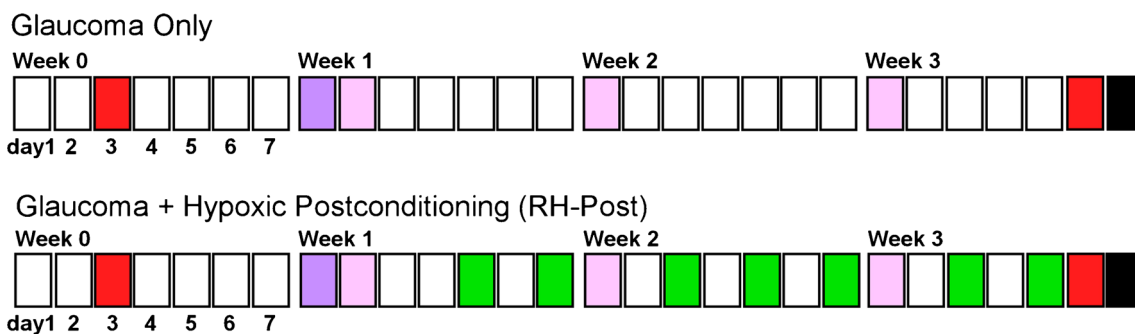


Fig. 1 Experimental protocol. Schematic timeline defining the experimental protocol employed in both study groups. The initial episcleral vein ligation surgery (dark purple) was performed on day 1 of week 1; the 24-h, 1-week, and 2-week follow-up surgeries (light purple) are shown. Flash visual-evoked potential and optometry functional metrics (red) were performed 4 days prior to the initial episcleral vein

ligation surgery, and the day before the last day of the 3-week period of experimental glaucoma (black), when animals were sacrificed for morphological assessments of retinal ganglion cell soma and axon loss. In the repetitive hypoxic postconditioning (RH-Post) group, mice were exposed for 1 h to systemic hypoxia (11 % oxygen) on the 7 days shown (green) prior to being killed

Functional Analyses of Glaucomatous Injury and RH-Post-mediated Protection

Flash Visual-Evoked Potentials

We recorded flash visual-evoked potentials (fVEPs), as described previously [13, 14], using a UTAS Visual Electrodiagnostic Testing System with BigShot™ Ganzfeld (LKC Technologies, Gaithersburg, MD, USA). In brief, mice were anesthetized with ketamine/xylazine, the pupil was dilated with 1 % atropine, corneal anesthesia was applied (procaïne, 0.5 %), followed by hydration maintenance with 2.5 % hypromellose (“Gonak,” Akorn, Lake Forest, IL, USA). Animals were placed on a thermoregulated heating pad and held in place by a tooth-holder headset; a 0.25-mm diameter platinum wire (AA45093BY; Fischer Scientific, Waltham, MA, USA) was inserted under the scalp for 1.0 cm over the mouse visual cortex and connected to a UBA-4200 digital biomedical amplifier using a “Mini SMD Grabber” test clip lead with jack (model 3782; Pomona Electronics, Everett, WA, USA). A reference needle electrode was placed through the pinna of one ear, and a ground electrode inserted under the skin at the base of the tail. Bilateral fVEP responses were recorded with both eyes open, and both eyes covered with hard contact lenses painted with black nail polish; then, unilateral fVEPs were recorded with the experimental eye or the control eye covered. Resting periods of 5 min were instituted before and between each recording, during which the room was darkened; recordings themselves were obtained under very dim room light. Flash parameters consisted of 0.2 lg (cd-s/m²) white flashes (≤5 ms duration) presented on a dark background, delivered via the Ganzfeld sphere; responses to 80 consecutive flashes presented at 1.9 Hz were averaged for each trial. After testing, animals received topical antibiotic and were allowed to recover in a 37-°C warming chamber until ambulatory, then returned to their home cages. Three distinct amplitudes and latencies from the characteristic fVEP

waveform (see Fig. 6A for representative trace showing 0–N1, P1–N1, and N1–P2) were manually quantified for both eyes of each mouse in a blinded fashion.

Visual Acuity by Optokinetics

Visual acuity (VA) was assessed in conscious mice by optokinetics, using a commercially available apparatus and the associated software (OptoMotry; Cerebral Mechanics, Lethbridge, AB, Canada), as described previously [15, 16]. Briefly, mice were placed on a platform in a closed chamber with a projection of virtual cylinders of varying spatial frequency at 100 % contrast. The spatial frequency of the rotating columns was recorded when the head movement of the mouse responding to the rotating columns was observed. VA was defined the highest spatial frequency, in cycles/degree (c/d), to which a given mouse responded. Changing the rotational direction of the cylinder (i.e., clockwise for the left eye and counterclockwise for the right eye), provided independent assessments of the VA of each eye. Baseline VA was determined bilaterally within a week before performing the initial episcleral vein ligation procedure to elevate IOP; VA was then measured again after 3 weeks of experimental glaucoma.

Structural Analyses of Glaucomatous Injury and RH-Post-mediated Protection

Axon integrity was quantified in the postlaminal optic nerve by confocal immunofluorescence microscopy of SMI32-immunopositive axons, as described previously by us and others [10, 11, 17–19]; SMI32 has been shown to stain the medium and large, alpha-subtype of RGCs [20, 21], which, while not inclusive of all RGCs, are found evenly distributed throughout the retina [20]. In brief, after obtaining fVEP and optokinetic measurements in untreated and RHP-treated mice after 3 weeks of experimental glaucoma, animals were euthanized by transcardial perfusion, the retina was removed and

flat-mounted (see below), and an optic nerve segment from the postlamina region (50–150 μm behind the globe) was fixed overnight in 4 % paraformaldehyde, rinsed three times with phosphate buffered saline, transferred to 10 % formalin, and then paraffin-embedded. Four- μm -thick sections perpendicular to the long axis of the nerve were collected, deparaffinized, rehydrated, and subjected to antigen retrieval by immersing the tissue for 10-min in boiling “antigen unmasking solution” (H3300; Vector Laboratories, Burlingame, CA, USA). After a 20-min incubation in blocking buffer, sections were incubated overnight at 4 °C with primary antibodies against the nonphosphorylated neurofilament heavy subunit SMI-32 (1:1000, mouse monoclonal; Covance, Emeryville, CA, USA), followed by incubation at room temperature for 1 h with secondary antibody [1:400, goat anti-mouse IgG linked to Alexafluor 488, A11001; Molecular Probes (Eugene, OR, USA) for SMI-32]. VectaShield Hardset Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories) was used for counterstaining nuclei. Confocal images [LSM 5 PASCAL; 40 \times objective (1.25 NA); Carl Zeiss, Jena, Germany] were obtained at excitation wavelengths of 405, 488, and 560 nm. Total fluorescence intensities of SMI32-positive axons within the nerve border were determined by binarizing the image after background subtraction to identify the total surface area in the nerve covered by SMI32 above threshold, calculating the total fluorescence intensity of the resulting image, and then normalizing to the total area of SMI32-positive signal, as detailed previously [10, 11].

RGC soma viability analyses were undertaken using NeuN immunohistochemistry on retinal flat mounts, as described previously by us and others [10, 11, 22–24]. NeuN is a well established RGC-specific antigen not found in inner nuclear layer cells [25]; thus, antibodies to NeuN are widely used to differentiate RGCs from displaced amacrine cells in the ganglion cell layer, with a false positive rate when quantified in young mice of no more than 5 % [23]. Briefly, harvested retinæ were washed 3 times in Tris-buffered saline and Tween 20 (TBST) buffer, and permeabilized for 30 min with 0.3 % Triton X-100 (T-9284; Sigma, St. Louis, MO, USA) in TBS buffer. After blocking nonspecific binding with 1 % bovine serum albumin (Jackson ImmunoResearch, West Grove, PA, USA), 0.15 % Triton X-100, and 10 % normal goat serum (Jackson ImmunoResearch) in TBST for 30 min at room temperature, retinæ were incubated overnight at 4 °C with NeuN [1:500, monoclonal anti-NeuN (MAB377); Chemicon/Millipore, Billerica, MA, USA]. Following TBST wash, secondary antibody [1:400, goat antimouse IgG linked to Alexafluor 568 (A11004); Invitrogen] was applied for 1 h at room temperature. Thereafter, retinæ were flat-mounted with 50 % glycerol (G-5516; Sigma) and coverslipped.

Twelve distinct regions of peripheral retina (3 per quadrant) were examined by confocal fluorescence microscopy (LSM 5

PASCAL, 20 \times objective, 0.5 NA, excitation wavelength= 560 nm; Carl Zeiss). A blinded observer initially established fluorescence thresholds for each experimental/fellow eye pair and applied manual corrections of scanned images to remove signal artifacts; thereafter, AnalySIS software (Soft Image System, Münster, Germany) was used to count automatically NeuN-positive RGC soma, defined as all homogeneously stained and round-in-shape NeuN-positive cell bodies. Resultant counts were analyzed by quadrant, as well as across the entire retina.

Statistics

All results are provided as means \pm SEM. Data in Figs. 6, 7, 8, and 9 were normalized to the respective values measured in the fellow eye of the untreated control group, so that potential effects of RH-Post on the nonglaucomatous fellow eye, if any, would be revealed. Nonparametric signed rank and rank sum tests were used to identify significant differences between measures from paired eyes in the same animal, and from eyes in different animal groups, respectively, using raw data. Quadrant-to-quadrant comparisons of RGC soma numbers within animals (experimental *vs* fellow eye) and between animals (untreated *vs* RH-Post-treated) were identified by repeated measures analysis of variance on ranks followed by the Tukey's test. IOP comparisons within and between animals in both experimental groups over the 3-week period of intraocular hypertension were identified by one-way repeated measures analysis of variance. In all instances, $p < 0.05$ was accepted as significant.

Results

IOP

As we found previously in this model [9–11], sustained increases in IOP were achieved secondary to episcleral vein ligation. Figure 2 shows IOP changes measured in both eyes in mice from the control and RH-Post groups. There were no significant differences in baseline IOP [10.4 ± 0.5 and 11.4 ± 0.5 mmHg for the fellow eyes of the control ($n=16$) and RH-Post ($n=11$) groups, respectively, and 10.4 ± 0.5 to 11.7 ± 0.6 mmHg in experimental eyes of the control and RH-Post groups, respectively] between the experimental groups. The increases in IOP measured in the experimental eyes were significantly greater ($p < 0.001$ to $p = 0.002$) at each of the 4 time points than the respective baseline IOP value for that eye, and significantly greater (all $p < 0.001$) than the respective IOP in the corresponding fellow eye at the same timepoint. IOPs in the experimental group ranged from 17.6 ± 1.0 to 17.6 ± 1.7 mmHg at 24 h, from 21.4 ± 1.0 to 24.4 ± 1.7 mmHg at

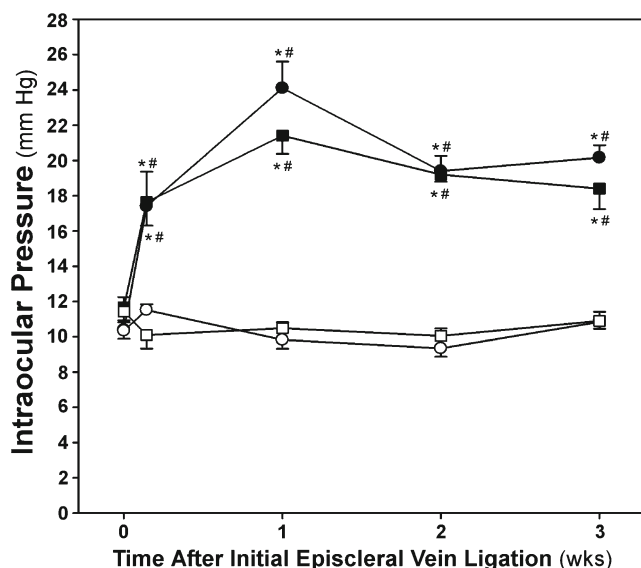


Fig. 2 Intraocular pressure (IOP) in untreated mice and mice with repetitive hypoxic postconditioning (RH-Post). IOP shown for experimental eyes (filled symbols) and respective fellow eyes (open symbols) in untreated (squares; $n=16$) and RH-Post-treated (circles; $n=11$) mice. At 24 h, and 1, 2, and 3 weeks, all IOP values for the experimental eyes in each group were significantly different from their respective baselines ($*p<0.05$) and from their fellow eyes at each time point ($^{\#}p<0.05$). There was no statistically significant difference in experimental IOP values (mean \pm SEM) between the untreated mice and mice with RH-Post

1 week, from 19.2 ± 0.4 to 19.4 ± 0.9 mmHg at 2 weeks, and from 18.4 ± 1.2 to 20.1 ± 0.8 mmHg at 3 weeks of sustained intraocular hypertension. There were no statistically significant differences in the extent of IOP elevation in the experimental eyes among both groups at any time point. In addition, there were no systematic differences in the number of episcleral vein ligations performed in a given animal and its corresponding IOP, or between the experimental eyes of the untreated and RH-Post-treated mice. In sum, the experimental eyes in our study started with the same IOPs and experienced the same elevation in IOP after episcleral vein ligation, independent of whether the mice received the RH-Post treatment or not.

Validation of Lack of Ischemic Injury Secondary to Episcleral Vein Ligation

As shown in the hematoxylin and eosin-stained cross-sections of mid-central retina shown in Fig. 3, there was no evidence of ischemia-induced cell loss in the inner nuclear (and outer nuclear) layers, or thinning of these layers or of the inner or outer plexiform layers, at any time (24 h, 1 week, or 3 weeks) after IOP elevation, unlike what we have consistently witnessed previously in this strain 1 week after a 30–45-min period of complete retinal ischemia induced by elevation of IOP to above systolic blood pressure [6, 8].

In addition to hematoxylin and eosin staining, we also performed double immunohistochemical labeling of NeuN-, caspase-3-, and caspase-9-positive ganglion cells in cross-sections of retina from mice in which episcleral vein ligation was performed 24 h, 1 week, and 3 weeks previously [22–24]. As shown in Figs 4 and 5, the retinæ look completely normal, with no evidence of cleaved caspase-9 or cleaved caspase-3 expression in any cell layer at any time after elevating IOP, other than in the ganglion cell layer at later time points, where and when glaucomatous cell death is to be expected. Specifically, in neither the central nor peripheral retina of glaucomatous eyes did we find cells in the ganglion cell layer that were immunopositive for either enzyme at 24 h, and only for cleaved caspase-3 could we find a very rare, immunopositive cell in the ganglion cell layer of the peripheral retina at 1 week (Fig. 5). After 3 weeks of experimental glaucoma, occasional cleaved caspase-9-positive cells (Fig. 4) and occasional cleaved caspase-3-positive cells (Fig. 5) could be found in the ganglion cell layer of the peripheral retina. These caspase-9- and caspase-3-positive cells were always co-localized with NeuN-positive cells, as shown in the individual panels and in the merged photomicrographs in Figs. 4 and 5, supporting their identity as RGCs. Interestingly, the caspase-9- and caspase-3-positive cells did not co-localize with the

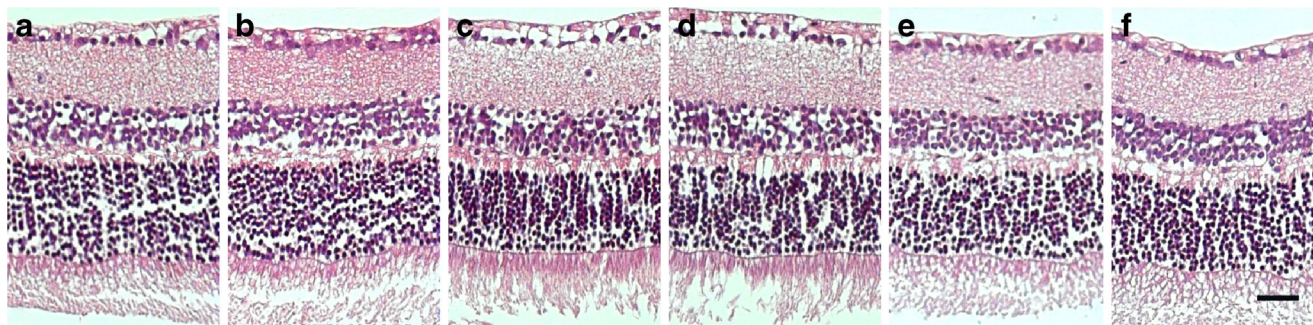


Fig. 3 Lack of ischemic injury following episcleral vein ligation. Hematoxylin and eosin-stained cross-sections of mid-central retinae from (B, D, F) glaucomatous and (A, C, E) matched fellow eyes obtained (A, B) 24 h, (C, D) 1 week, and (E, F) 3 weeks after

intraocular pressure elevation by episcleral vein ligation. Note, no evidence of ischemic injury in the inner or outer retina is present at any time. Scale bar=50 μ m

Fig. 4 Cleaved caspase-9 immunohistochemistry after episcleral vein ligation. Cleaved caspase-9 immunohistochemistry in representative retinæ from experimental glaucoma (GL) eyes and fellow eyes obtained (A) 24 h (upper), (A) 1 week (lower), and (B) 3 weeks after episcleral vein ligation. Cleaved caspase-9 (green) shown with NeuN (red) for retinal ganglion cell (RGC) identification, 4',6-diamidino-2-phenylindole (DAPI; blue) to mark nuclei, and merged images. Note, only after 3 weeks of experimental glaucoma were caspase-9-positive cells apparent across the retina, and only then in a cytoplasmic location in some cells in the ganglion cell layer, identified as RGCs by co-immunostaining with NeuN (arrowheads). Scale bar (shown in Fig. 5)=30 µm

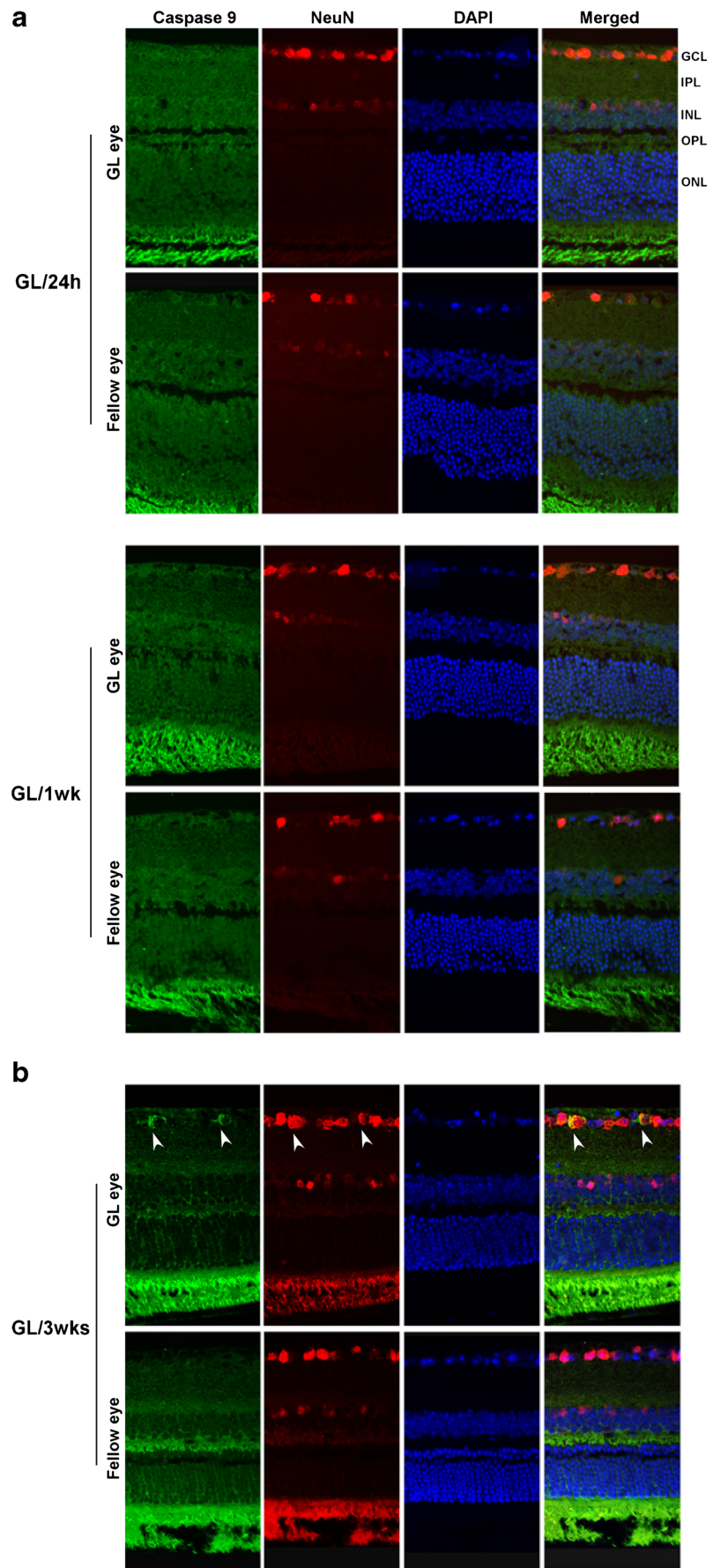
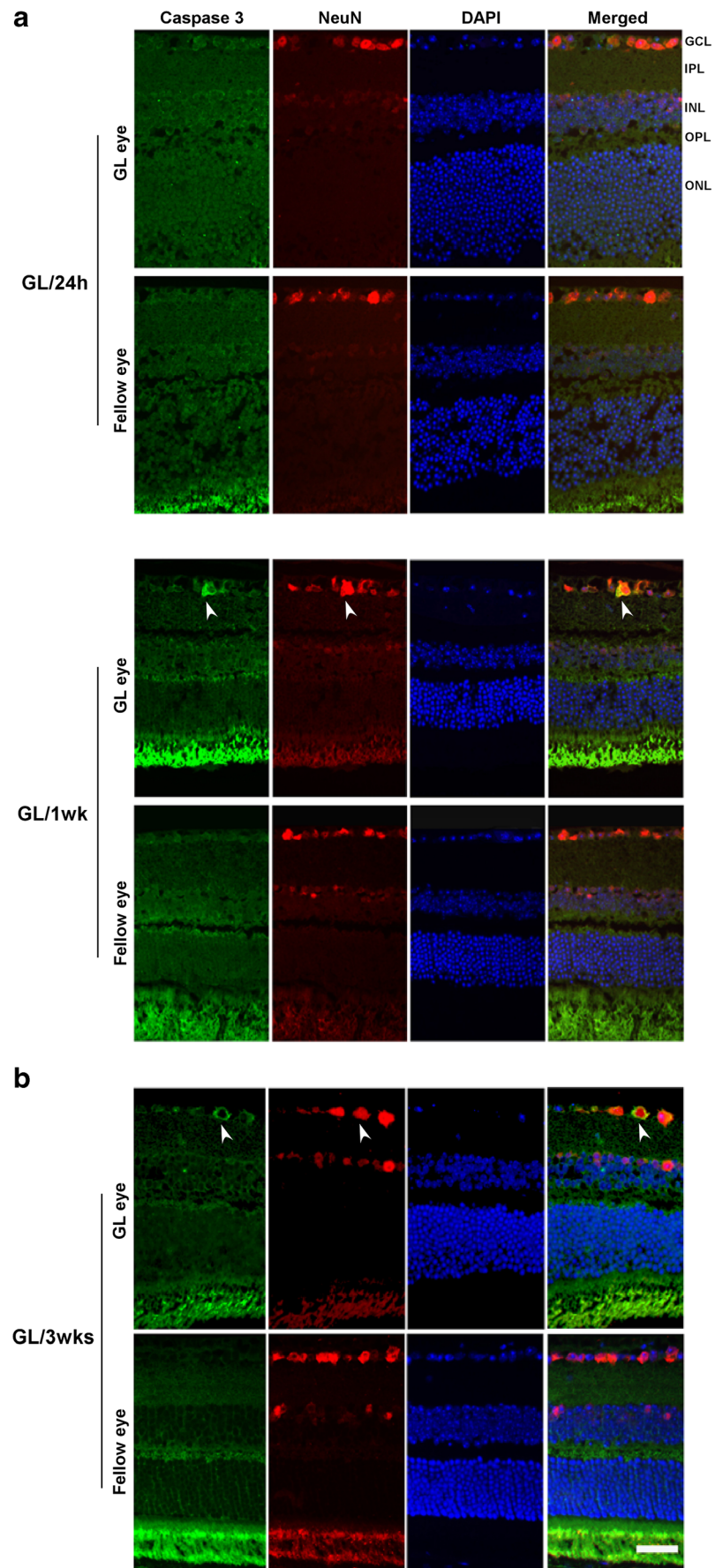


Fig. 5 Cleaved caspase-3 immunohistochemistry after episcleral vein ligation. Cleaved caspase-3 immunohistochemistry in representative retinæ from experimental glaucoma (GL) eyes and fellow eyes obtained (A) 24 h (upper), (A) 1 week (lower), and (B) 3 weeks after episcleral vein ligation. Cleaved caspase-3 (green) shown with NeuN (red) for retinal ganglion cell (RGC) identification, the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; blue), and merged images. Note, caspase-3-positive cells were not evident at 24 h, but cytoplasmic staining was seen occasionally 1 or 3 weeks after episcleral vein ligation in the retinal ganglion cell layer, in cells co-immunopositive for the RGC marker NeuN (arrowheads). Scale bar=30 μ m



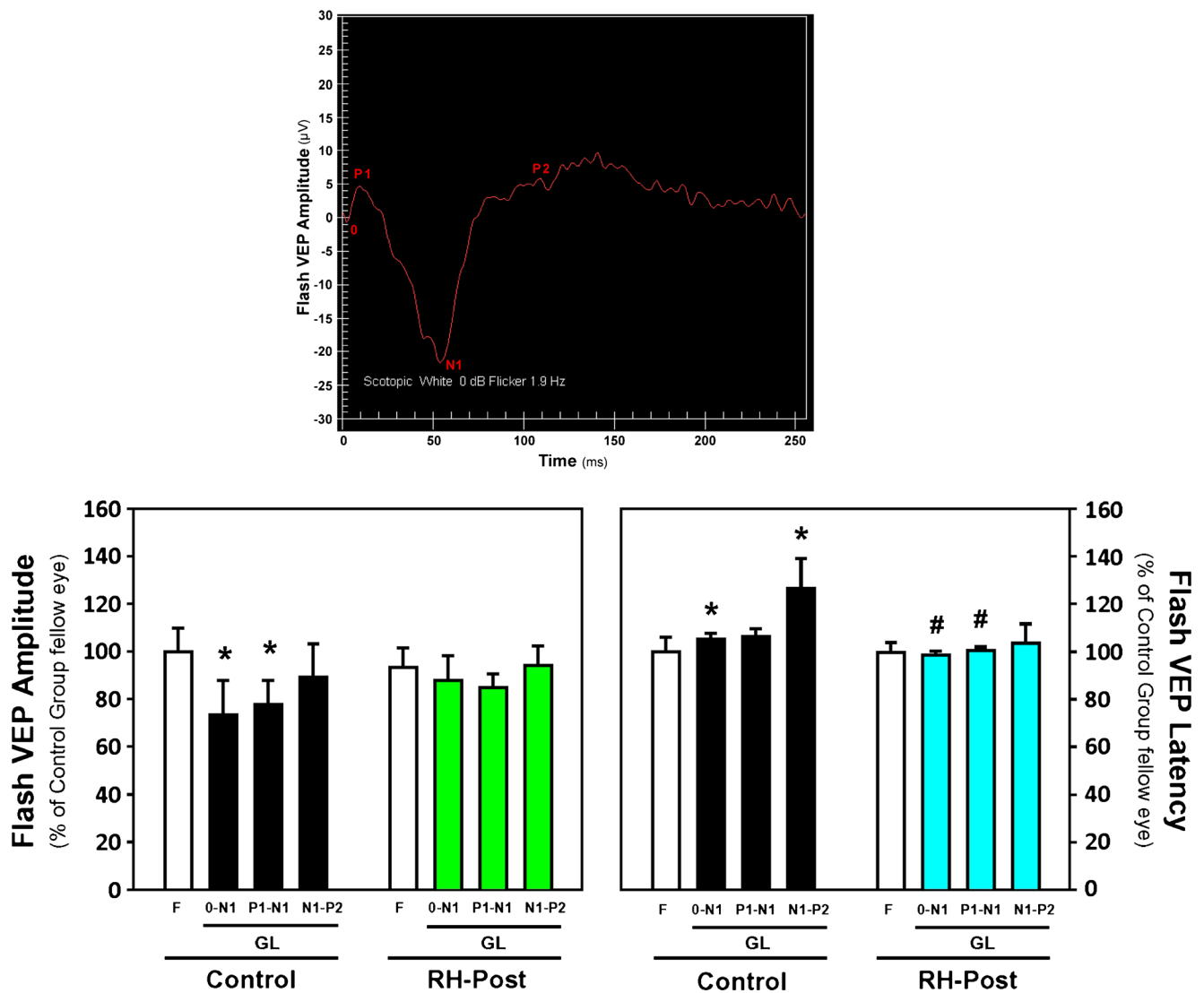


Fig. 6 Flash visual-evoked potential (fVEP) amplitudes and latencies in untreated control mice, and mice with repetitive hypoxic postconditioning (RH-Post). (A) Representative fVEP waveform showing the characteristic locations of the P1, N1, and P2 peaks from which the 0–N1, P1–N1, and N1–P2 amplitudes and latencies were quantified. Left histogram: 0–N1, P1–N1, and N1–P2 amplitudes of the flash VEP waveforms in untreated control mice ($n=9$; black bars) and mice with RH-Post ($n=11$; green bars) measured after 3 weeks of

experimental glaucoma (GL) and normalized to amplitudes measured in fellow (F) eyes of the control group. Right histogram: 0–N1, P1–N1, and N1–P2 latencies of the fVEP waveforms in untreated control mice (black bars) and mice with RH-Post (blue bars) measured after 3 weeks of GL and also normalized to latencies measured in F eyes of the control group. Mean±SEM. * $p<0.05$ vs amplitude or latency in respective fellow eye; # $p<0.05$ vs respective amplitude or latency in experimental eye of matched control group

nuclear-specific marker DAPI, suggesting that the nucleus of these apoptosis-undergoing RGCs may have already been destroyed or at least rendered nonimmunoreactive for DAPI during the apoptotic process. Finally, performing TUNEL staining on our 3-week retina samples showed occasional TUNEL-positive cells in the RGC layer of the peripheral retina in mice with 3 weeks of experimental glaucoma, but not elsewhere in the retina (photomicrographs not shown). Taken together, these results indicate that our episcleral vein ligation method for inducing sustained elevations in IOP do not cause retinal ischemia.

fVEP

A representative fVEP trace is provided, with reference waveform peaks identified (Fig. 6). At baseline, the three fVEP waveform amplitudes did not differ significantly ($p=0.597$, $p=0.860$, and $p=0.944$, respectively) between control ($n=9$) and experimental ($n=11$) groups. Figure 6 shows that, in untreated control mice, the 0–N1 and P1–N1 amplitudes of the fVEP waveform were reduced $27\pm14\%$ ($p=0.016$) and $22\pm10\%$ ($p=0.008$), respectively, relative to fellow eyes after 3 weeks of experimental glaucoma; the N1–P2 amplitude

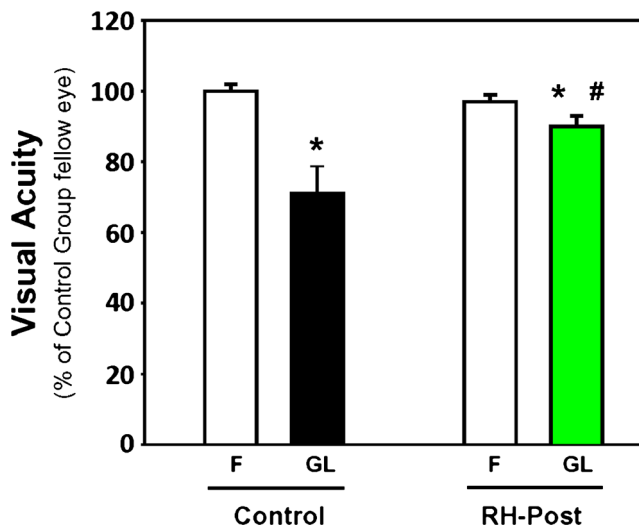


Fig. 7 Visual acuity (VA) in untreated control mice and mice with repetitive hypoxic postconditioning (RH-Post). VA in control mice ($n=10$; black bar) and mice with RH-Post ($n=11$; green bar), measured by optometry after 3 weeks of experimental glaucoma (GL) and normalized to VA in fellow (F) eyes of the control group. Mean \pm SEM. * $p<0.05$ vs respective fellow eye; # $p<0.05$ vs matched control group

remained statistically unchanged (reduced $11\pm 14\%$). However, in RH-Post mice, the extent of disease-induced fVEP amplitude reduction was attenuated, with 0–N1 and P1–N1 amplitudes reduced only $9\pm 5\%$ and $6\pm 4\%$, respectively; both waveform amplitudes were not significantly different ($p=0.175$ and 0.102 , respectively) from those measured in the fellow eye. This represented a degree of protection of

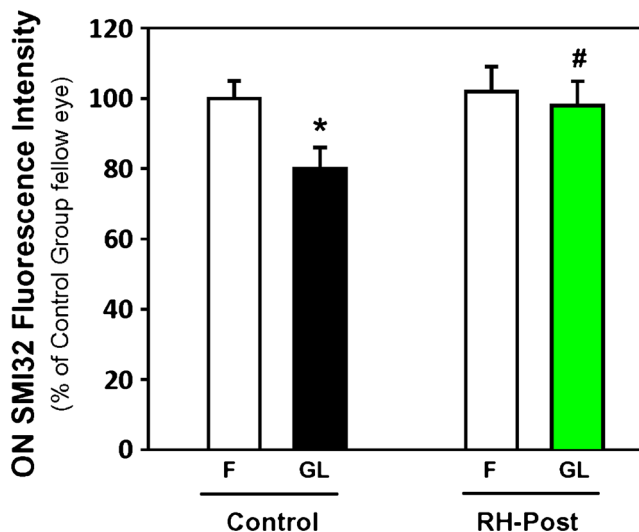


Fig. 8 SMI32 fluorescence intensity in postlamina optic nerve (ON) of untreated control mice, and mice with repetitive hypoxic postconditioning (RH-Post). Fluorescence intensity, normalized to levels in the fellow (F) ON of the control group in control mice ($n=12$; black bar) and mice with RH-Post ($n=9$; green bar) measured after 3 weeks of experimental glaucoma (GL). Mean \pm SEM. * $p<0.05$ vs respective fellow eye; # $p<0.05$ vs matched control group

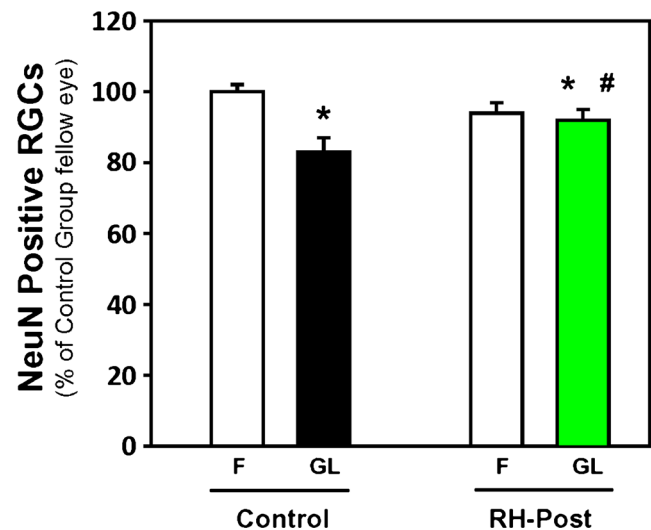


Fig. 9 Total NeuN-positive retinal ganglion cell (RGC) soma in peripheral retinae from untreated control mice and mice with repetitive hypoxic postconditioning (RH-Post). NeuN-positive RGC soma in 12 regions of peripheral retina were quantified and normalized to numbers measured in the fellow (F) retina of the control group in control mice ($n=13$; black bar) and mice with RH-Post ($n=11$; green bar) after 3 weeks of experimental glaucoma (GL). Mean \pm SEM. * $p<0.05$ vs respective fellow eye; # $p<0.05$ vs matched control group

66% ($p=0.068$) and 71% ($p=0.058$), respectively, for these metrics of visual performance.

fVEP waveform latencies did not differ significantly ($p=0.469$, $p=0.849$, and $p=0.732$, respectively) between control ($n=9$) and experimental ($n=11$) groups. Also shown in Fig. 6, in the untreated controls subjected to 3 weeks of intraocular hypertension, the duration of the 0–N1 and N1–P2 waveforms were significantly prolonged by $5\pm 3\%$ ($p=0.039$) and $26\pm 12\%$ ($p=0.004$); the duration of the P1–N1 waveform was lengthened $6\pm 3\%$, but this did not reach statistical significance ($p=0.148$). In RH-Post mice, however, the latencies defining the fVEP response after 3 weeks of disease were no different ($p=0.120$ – 0.700) than those measured in fellow eyes, and two of the three latency periods (0–N1 and P1–N1) were significantly shorter ($p=0.020$ and $p=0.007$, respectively) than those same metrics in the control mice not receiving RH-Post.

VA

Baseline VA did not differ significantly between eyes in animals from the untreated control group ($p=0.651$; $n=10$) nor between eyes in animals from the RH-Post-treated group ($p=0.969$; $n=11$). Baseline VA in the fellow eyes of the control group was not significantly different ($p=0.309$) from that in the RH-Post group. Figure 7 shows that, in response to 3 weeks of intraocular hypertension, VA in untreated control mice was reduced $29\pm 8\%$ ($p<0.001$) relative to fellow eyes, whereas in mice receiving RH-Post ($n=11$), VA was only

reduced $7\pm 3\%$ [but still significantly lower ($p=0.042$) than that measured in fellow eyes]. Relative to the control mice, this represented a significant, 66 % improvement ($p=0.004$) in VA as a result of the RH-Post treatment.

RGC Axon Viability

There was no statistically significant difference ($p=0.749$) in postlaminar SMI32 fluorescence, a metric of axon integrity, between the fellow eyes of the untreated control group ($n=12$) and RH-Post group ($n=9$). As shown in Fig. 8 and consistent with our previous studies [9–11], 3 weeks of experimental glaucoma resulted in a significant loss of SMI32 in the postlaminar optic nerve, as revealed by quantitative SMI32 immunohistochemistry. Specifically, a $20\pm 6\%$ ($p<0.001$) reduction in postlaminar SMI32 fluorescence intensity was measured in the experimental eyes of the untreated controls relative to their fellow eyes. However, in animals treated with RH-Post, postlaminar SMI32 fluorescence was only reduced $4\pm 3\%$ [a change not significantly different ($p=0.301$) from fellow eyes]. Thus, with respect to this structural metric of axon loss, RH-Post protected 80 % ($p=0.043$) of axons in the postlaminar optic nerve that would have been lost without treatment.

RGC Soma Viability

There was no statistically significant difference ($p=0.183$) in the number of NeuN-positive RGC soma between the fellow eyes of the untreated control group ($n=13$) and RH-Post group ($n=11$). Figure 9 shows that, as with axonal dropout, 3 weeks of experimental glaucoma also resulted in significant RGC soma loss, as revealed by quantitative NeuN immunohistochemistry. Consistent with what we reported previously in this model using NeuN or other RGC-specific markers [9–11], overall RGC soma counts across the peripheral retina were reduced $17\pm 4\%$ ($p<0.001$) in the experimental eyes of untreated mice, relative to fellow eyes. RGC soma loss was significant across all quadrants, with reductions of 21 % ($p=0.006$ vs fellow eye), 16 % ($p=0.010$), 17 % ($p<0.001$), and 16 % ($p<0.001$) in the dorsal-nasal, ventral-nasal, dorsal-temporal, and ventral-temporal quadrants, respectively (data not shown). In the experimental eyes of mice that received RH-Post ($n=11$), however, overall RGC soma counts were only reduced $2\pm 1\%$ ($p=0.014$ vs fellow eye), indicating an overall rescue of 88 % ($p<0.001$) of RGC soma by RH-Post.

By quadrant, RGC soma number in the experimental eyes of RH-Post mice was not significantly different from the corresponding quadrant in the fellow eyes, suggestive of complete protection across all quadrants (data not shown). Specifically, in RH-Post mice, we measured a 12 % ($p=$

0.978) loss in the dorsal-nasal quadrant and a 4 % ($p=0.365$) loss in the ventral-temporal quadrant; RGC soma numbers in the ventral-nasal and dorsal-temporal quadrants were actually 3 % ($p=0.278$) and 2 % ($p=0.966$) higher than those in the corresponding quadrants of the fellow eye. Comparing RGC soma numbers by quadrant between untreated controls and RH-Post mice revealed that the extent of rescue was 42 % ($p=0.297$) for dorsal-nasal, 119 % ($p=0.011$) for ventral-nasal, 112 % ($p=0.183$) for dorsal-temporal, and 75 % for ventral-temporal ($p=0.706$); note, only the ventral-nasal quadrant exhibited statistically significant protection. Collectively, these findings indicate that RGC soma loss can be consistently detected within animals when analyzed by quadrants, but documenting RGC soma protection between animals as a result of RH-Post treatment is best evidenced by quantifying RGC soma numbers across the entire peripheral retina.

Discussion

The results of the present study in an inducible mouse model of open-angle glaucoma reveal that repetitive hypoxic postconditioning during the initial 3 weeks of this neurodegenerative disease significantly abrogates functional and morphologic metrics of RGC injury/death. Specifically, glaucoma-induced reductions in overall visual performance (as assessed by fVEPs), VA (determined by optokinetic tracking), RGC soma number in the peripheral retina (by NeuN immunohistochemistry), and RGC axon integrity at the level of the postlaminar optic nerve (by SMI32 immunohistochemistry)—the extent of which were collectively indicative of mild-to-moderate disease in untreated mice—were all robustly blocked in animals treated with RH-Post. These findings indicate that epigenetic responses capable of protecting RGCs can be induced in a sustained fashion after disease onset, and suggest that these pleiotropic, survival-enhancing mechanisms could serve as novel therapeutic targets for patients with glaucoma.

To date, the use of both preconditioning and postconditioning strategies to activate endogenous mechanisms of cytoprotection are widespread in the CNS literature, dating back to the early 1990s; however, these studies have been confined almost exclusively to acute injury paradigms. In these settings, the adaptive response induced by the conditioning stimulus is short-lasting, abrogating the extent of an injury process that, while severe, typically ‘matures’ within hours or a couple of days. Our discovery that repetitive presentations of the conditioning stimulus could extend the duration over which both retina and brain could resist acute ischemic injury [8, 26], a finding later validated by others in models of acute ischemic brain injury [27–30], carried the implicit assumption that the long-lasting, prosurvival phenotype induced epigenetically by repetitive conditioning might also

protect against chronic disease. Indeed, we showed that six short bouts of systemic hypoxia over a 2-week period prior to the onset of experimental glaucoma reduced the resultant loss of RGC soma and axons that occurred over the subsequent 10 weeks of disease [9]. Despite this success, given that the clinical applicability of preconditioning against future disease seems limited, we undertook the present study of postconditioning efficacy.

While postconditioning against acute brain injury, using a variety of postconditioning stimuli and a number of injury endpoints, is well established, postconditioning for ophthalmologic disorders is not. For acute retinal ischemic injury, postconditioning with brief ischemia or carbon monoxide has proven efficacious [31–34], as has environmental enrichment [35]. Studies of postconditioning for more chronic retinal injury paradigms, however, are very limited [36, 37]. That repetitive postconditioning might provide a viable treatment strategy for glaucoma is supported by two preclinical studies to date. One is our previous study showing RGC soma and axon protection in the same inducible mouse model of glaucoma by intermittent hypoxic preconditioning before disease onset [9]. The second reports functional and histological metrics of protection in rats postconditioned weekly with brief local ischemia during the period of intraocular hypertension [38]. Our current observations extend these latter findings in several ways. First, we show that exposing conscious animals to intermittent systemic hypoxia, a noninvasive postconditioning stimulus, can promote glaucoma resistance without the requirement for inducing repetitive anesthesia and/or repetitive, direct retinal ischemia. Second, we demonstrate postconditioning efficacy in a model of glaucoma exhibiting progressive, time-dependent RGC loss secondary to IOP elevation by a relatively common episcleral vein occlusion approach [9–11, 39–44], in contradistinction to a more obscure model dependent on weekly anterior chamber injections of chondroitin sulfate to elevate IOP [38]. Third, we now document that glaucomatous protection by postconditioning is feasible in mice. Fourth, although both studies histologically quantified significant improvements in soma survival and axon integrity in postconditioned animals, we provided more extensive fVEP amplitude and latency metrics, and we also measured postconditioning-induced enhancements in VA. Independent of model and methodologies, our findings indicate that postconditioning-based treatments, repeated at defined intervals during the progression of disease, offer the potential to significantly abrogate glaucomatous damage.

Generally speaking, quantitative metrics of visual performance reflect the extent of both injury and protection that can only be inferred by morphologic analyses; moreover, functional alterations are often seen in glaucoma in the absence of gross changes in retinal cell structure [45, 46], resulting in nonlinear structure-function relationships [47, 48]. In our model, impairments in visual function and performance to levels of about 70–75 % of fellow eye controls, concomitant

with optic nerve axon integrity and soma survival on the order of 80–85 % of fellow eye controls, are indicative of mild-to-moderate injury after 3 weeks of sustained IOP elevation. Importantly, RH-Post was efficacious in abrogating all of these RGC injury metrics in the present study, and confirms that improvements in visual performance are feasible with this therapy.

We also advanced findings in the present study corroborating our claim that, in our glaucoma model, RGCs are not dying from ischemia secondary to vein ligation-induced choroidal damage, but from sustained elevations in IOP. This is likely the result of the fact that no penetration of the sclera occurs when we ligate the episcleral veins, thereby avoiding damage to the underlying choroidal tissue secondary to the focal heating caused by cautery or laser treatments [41, 49, 50]. Our extensive histological examinations of the retina revealed no evidence of changes reflecting even the mildest of ischemic injury in the inner or outer nuclear layers, nor any shrinkage of overall retinal thickness, at any postischemic time—changes that would be expected with retinal ischemia. Moreover, at no postischemic time point could we find cleaved caspase-9- or caspase-3-positive cells, or TUNEL-positive cells, in any layer other than the ganglion cell layer in glaucomatous eyes, indicating that no ischemia-induced apoptotic changes in non-RGC cells resulted from vein ligation. Other investigators employing a similar episcleral vein occlusion approach for IOP elevation in mice and rats also did not report morphologic evidence of inner retinal injury nor changes in retinal blood vessels [40–44, 49, 51–57]. Moreover, changes in the extent of reduction of ganglion cell-dependent positive and negative scotopic threshold response (pSTR and nSTR, respectively) amplitudes in mice with laser ablation of episcleral veins directly correlated with the extent of relative IOP elevation [51]. Thus, the findings we report herein, together with the extensive accumulated evidence of others, are consistent with the loss of RGCs secondary to sustained elevations in IOP and not ischemia.

The significant caveat of the present study is that the mechanistic basis for such robust functional/structural protection remains unexplored at this juncture. Given that the progressive RGC injury occurring in glaucoma is characterized by a multifactorial pathology putatively involving a host of excitotoxic, inflammatory, immune, vascular, biomechanical, and other factors [58–60], and that the mechanisms dictating RGC soma and RGC axon loss may be quite distinct [61–63], the epigenetic changes induced by RH-Post will likely be extensive in order to account for the magnitude of the protection achieved. It is also worth noting that, although exposures to intermittent systemic hypoxia may not ultimately be adopted as a clinical treatment for glaucoma patients, this very stimulus has established a strong record for protection against a variety of acute and chronic diseases in the CNS [27–30, 64–67], and other tissues [68–70], and may not be easily mimicked by a pharmacologic, monotherapy approach.

In conclusion, the functional and structural findings presented herein document that postconditioning during disease can robustly reduce the progressive glaucomatous injury of RGCs and the attendant vision loss. Further studies to elucidate the proximal molecular mediators of this hypoxia-driven response may inform possible therapeutic agents capable of protecting as robustly as intermittent hypoxia did here. But our study makes clear that an epigenetics-based therapeutic strategy can provide a viable treatment approach for patients with glaucoma.

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