Neuroprotection of the Inner Retina Also Prevents Secondary Outer Retinal Pathology in a Mouse Model of Glaucoma

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PURPOSE. We examined structural and functional changes in the outer retina of a mouse model of glaucoma. We examined whether these changes are a secondary consequence of damage in the inner retina and whether neuroprotection of the inner retina also prevents outer retinal changes.

METHODS. We used an established microbead occlusion model of glaucoma whereby intraocular pressure (IOP) was elevated. Specific antibodies were used to label rod and cone bipolar cells (BCs), horizontal cells (HCs), and retinal ganglion cells (RGCs), as well as synaptic components in control and glaucomatous eyes, to assess structural damage and cell loss. ERG recordings were made to assess outer retina function.

RESULTS. We found structural and functional damage of BCs, including significant cell loss and dendritic/axonal remodeling of HCs, following IOP elevation. The first significant loss of both BCs occurred at 4 to 5 weeks after microbead injection. However, early changes in the dendritic structure of RGCs were observed at 3 weeks, but significant changes in the rod BC axon terminal structure were not seen until 4 weeks. We found that protection of inner retinal neurons in glaucomatous eyes by pharmacological blockade of gap junctions or genetic ablation of connexin 36 largely prevented outer retinal damage.

CONCLUSIONS. Together, our results indicate that outer retinal impairments in glaucoma are a secondary sequela of primary damage in the inner retina. The finding that neuroprotection of the inner retina can also prevent outer retinal damage has important implications with regard to the targets for effective neuroprotective therapy.

Keywords: glaucoma, bipolar cells, horizontal cells, neuroprotection, retinal ganglion cells
to identifying potential targets for effective neuroprotective therapy.

METHODS

Experimental Animals

Experiments were performed on C57BL/6 wild-type (WT) mice and on connexin36 knock-out (Cx36−/−) mice and their wild-type (Cx36+/+) littersmates, 3 to 4 months old and of either sex. The Cx36−/− and Cx36+/+ mice were derived from F2 C57BL/6:129SvEv mixed-background litters. All animal procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved by the institutional animal care and use committee at the State University of New York College of Optometry.

Induction of Elevated IOP

IOP was elevated by unilateral injection of polystyrene microbeads (Invitrogen, Carlsbad, CA, USA), 10 μm in diameter, into the anterior chamber. An equivalent volume of PBS was injected into contralateral eyes to provide control (sham) measurements. A second microbead injection was performed at week 4, which maintained elevated IOP for at least 8 weeks. All injections were performed on animals anesthetized with a ketamine/xylazine mixture and topical application of proparacaine. IOP measurements were performed weekly between 10 AM and 12 PM, to minimize effects of diurnal IOP variation. Six measurements were obtained per eye and averaged.

Blockade of Gap Junctions

The GJ blocker meclofenamic acid (MFA, 20 mg/kg/day; Sigma-Aldrich, St. Louis, MO, USA) was administered via subcutaneous osmotic minipumps (ALZET 2004; DURECT, Cupertino, CA), as described previously. Mice were anesthetized under isoflurane anesthesia, and pumps were subcutaneously implanted into or across retinas, as the analysis was performed with grayscale thresholding. All data were imported into SigmaPlot software (Systat Software, San Jose, CA, USA), and histograms were constructed.

Immunohistochemistry

The immunohistochemical methods have been described previously. Eyecups were fixed with 4% paraformaldehyde, cryoprotected, and embedded in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA, USA), and 10- to 15-μm-thick frozen sections were cut. The tissues were incubated with diluted primary antibodies overnight (sections) or for 48 hours (whole mounts), washed, and then incubated in secondary antibodies for 2 to 4 hours. Images of immunolabeled retinal sections or whole mounts were acquired using a compound microscope equipped with a charge-coupled device camera (Diagnosys, Lowell, MA). Responses were averaged over 40 to 50 trials for weak stimuli and fewer trials for stronger stimuli. Signals were amplified, filtered (1–300 Hz), and digitized at 1 kHz with a resolution of 0.1 μV.

Electroretinogram Recording

The scotopic ERG responses were recorded from both eyes simultaneously. Visual stimuli consisted of brief (5 ms) white Ganzfeld flashes ranging between −6.7 and 2.0 log scot cd·s/m², produced by an array of light-emitting diodes (Diagnosys, Lowell, MA). Responses were averaged over 40 to 50 trials for weak stimuli and fewer trials for stronger stimuli. Signals were amplified, filtered (1–300 Hz), and digitized at 1 kHz with a resolution of 0.1 μV.

Statistical Analysis

Data are presented as mean ± SEM. Sample sizes (retinas, eyes, mice) were determined on the basis of our previous studies. Sample sizes were determined using values of groups and common standard deviation. Calculations were performed using two-sided tests with alpha = 0.05 and power = 0.8. Samples were allocated to experimental groups according to genotype; therefore, there was no randomization. To compare two experimental groups, we used a two-tailed Student’s t-test. Comparisons between larger groups were analyzed using one-way analysis of variance followed by Tukey’s multiple comparison test. Values of P < 0.05 were considered statistically significant.

RESULTS

Changes in Bipolar Cell Structure and Function in Glaucomatous Eyes

Unilateral injections of microbeads made at weeks 0 and 4 resulted in a sustained elevation of IOP from 10 to 15 μm thick. Measurements were averaged, using at least three retinas for each condition. For measures of SMI32 labeling in flatmount retinas, three or four square areas (300 × 300 μm) in each quadrant in the mid-periphery were analyzed and averaged across four retinas each for control and experimental protocols. Image software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the number of pixels with immunolabels above background. The number of pixels positive for a marker was then divided by the total pixel number and presented as a percentage of covered area. This parameter was independent of differences in the intensity of label within or across retinas, as the analysis was performed with grayscale thresholding. All data were imported into ImageJ software (National Institutes of Health, Bethesda, MD, USA), and histograms were constructed.
FIGURE 1. Effect of elevated IOP on bipolar cell structure and function in the C57BL/6 WT mouse. (A) Intraocular injection of microbeads (Bead) at weeks 0 and 4 results in a sustained elevation of IOP throughout the 8-week period as compared with sham-injected control (Ctrl) eyes. ***P < 0.001 for all time points; n = 10 eyes/group. (B) Example recordings show the b-waves of scotopic ERGs from WT mice under control conditions (black) and at 8 weeks after the initial microbead injection (red). There was a significant decrease of the peak amplitude (C) and an elevation of the time-to-peak latency (D) of the b-wave in glaucomatous eyes as compared with controls. Data are presented as mean ± SEM. *P < 0.05 for all intensities; n = 5 eyes/group. (E–H) Confocal images of vertical sections from control and glaucomatous
retinas at 8 weeks after the initial microbead injection immunostained with anti-PKCα for rod BCs and anti-Chx10 for cone BCs (asterisks). Arrowheads in G and H show rod BC dendrites. Projection of eight images, z = 0.7 μm. (I, J) Histograms quantify the reduction of rod and cone BCs (per 300-μm lateral distance) in microbead-injected eyes, 8 weeks after initial injection, compared with controls. Data are presented as mean ± SEM. *P < 0.01; n = 5 eyes/group. (K) Histogram showing the density of rod BC axon terminals in IPL of glaucomatous retinas as compared with control eyes. Data are presented as mean ± SEM. **P < 0.01; n = 5 eyes/group. Scale bar: 10 μm in all panels. OPL, outer plexiform layer; INL, inner nuclear layer; IP, inner plexiform layer; GCL, ganglion cell layer.

Prior to complete cell loss, neurons show structural changes to dendrites and axon terminals.60,61 We have reported that αRGCs in glaucomatous mouse eyes that survive at 8 weeks show a significant loss of dendrites.62 We extended this finding by calculating the dendritic lengths of anti-SMI32–labeled αRGCs at weekly intervals after microbead injection. We found the first significant change in dendritic length at 3 weeks, a 40% reduction from control values (P < 0.01; n = 3–5 eyes/group) (Figs. 2I–2L, 2P). In contrast, the first significant loss of RBC axon terminals was a 20% reduction (P < 0.01; n = 3–5 eyes/group) at 4 weeks (Figs. 2B, 2D, 2F, 2H, 2O). Clearly, early structural changes of RGC dendrites preceded those of BC axon terminals.

Neuroprotection of the Outer Retina in Glaucoma

To test whether the BC changes were a consequence of prior degenerative changes in the inner retina, we leveraged the neuroprotective actions of GJ blockade or ablation reported in a number of retinopathies, including glaucoma.26,31,32,42–44 We showed previously that selective genetic deletion of connexin 36 (Cx36) protein largely prevented loss of RGCs and ACs, preserved optic nerve axons, and reduced reactive gliosis in a mouse model of glaucoma.25 Importantly, this neuroprotective action was not associated with a lowering of IOP.

We examined Cx36+/− and Cx36−/− mouse retinas under control and microbead injection conditions. The microbead injections elevated IOP for 8 weeks in eyes of Cx36+/− and Cx36−/− mice, similar to that described above for C57Bl/6 WT mice (Fig. 3A). Scotopic ERGs recorded from microbead-injected Cx36+/− eyes showed a significant reduction of the b-wave amplitude (P < 0.001; n = 5 eyes/group) (Fig. 3B). In contrast, we found no difference in b-wave amplitude or time to peak of Cx36−/− eyes under control conditions or 8 weeks after microbead injection (P > 0.5; n = 5 eyes/group for both parameters) (Figs. 3B–3D). We also found no change in the number of RBCs in Cx36−/− mouse eyes (P > 0.5; n = 5 eyes/group) (Figs. 3F, 3I), nor in the structure of their dendrites or axon terminals (P > 0.5; n = 5 eyes/group) (Figs. 3H, 3J). Likewise, there was no change in the number of BCs in microbead-injected Cx36−/− eyes (P > 0.1; n = 5 eyes/group) (Figs. 3F, 3K). Qualitatively similar protection was observed in microbead-injected eyes of Cx36−/− control mice treated with the GJ blocker MFA (data not shown). Thus, ablation or blockade of GJs, shown previously to protect inner retinal neurons,48 also prevented the deleterious effects of glaucoma on BCs.

Synaptic Changes Associated With Rod Bipolar Cells in Glaucomatous Retinas

We next examined changes in synaptic structures associated with BC dendrites and axon terminals in microbead-injected eyes. Retinas of WT mice were double immunolabeled for PKCα and anti-CtBP2, an established

Timing of Bipolar Cell Structural Changes in Glaucoma

We have reported that the first significant loss of RGCs and amacrine cells (ACs) occurs at 4 weeks after initial microbead injection.55 In the next series of experiments, we examined BC structure at weekly intervals after microbead injection to compare the time course with changes in the inner retina. The first significant loss of RBCs, a 13% reduction from control values (P < 0.05; n = 3–5 eyes/group), was at 4 weeks (Figs. 2A, 2C, 2E, 2G, 2M), whereas a significant loss of CBCs, a 12% reduction (P < 0.01; n = 3–5 eyes/group), first occurred at 5 weeks after microbead injection (Fig. 2N).
marker for photoreceptor synaptic ribbons. For the first 3 weeks following microbead injection, the dendritic architecture of RBCs appeared similar to that in controls (Figs. 4A–4C). This included processes projecting to CtBP2-positive, horseshoe-shaped presynaptic ribbons (Fig. 4B, inset). However, structural changes in the OPL were first observed at 4 weeks and progressed over the next 4 weeks (Figs. 4D–4F). These changes included a retraction of dendrites and a 27% ($P < 0.01$, $n = 3$ eye/group) reduction in CtBP2-positive synaptic ribbons (Fig. 4J), with the spared ribbons
FIGURE 3. Effect of elevated IOP on BC structure and function in the Cx36<sup>−/−</sup> mouse. (A) Intracocular injection of microbeads at weeks 0 and 4 resulted in a sustained elevation of IOP throughout the 8-week period as compared with sham-injected control eyes. ***P < 0.001 for all time points; n = 5 eyes/group. (B) Example recordings showing that at 8 weeks after initial microbead injection the b-wave of the scotopic ERG was reduced in Cx36<sup>++/++</sup> mouse eyes but not in Cx36<sup>−/−</sup> mice (C, D) Scatterplots show that there was no change in the amplitude or time-to-peak latency of the ERG b-wave in glaucomatous eyes as compared with control eyes from Cx36<sup>−/−</sup> mice. P > 0.5; n = 5 eye/group. (E–H) Confocal images of vertical sections from control and glaucomatous retinas at 8 weeks after initial microbead injection of Cx36<sup>−/−</sup> mice immunolabeled with anti-PKCα for rod BCs and anti-Chx10 for cone BCs (asterisks). Arrowheads show the preservation of rod BC dendrites in the OPL of microbead-injected Cx36<sup>−/−</sup> retinas. Projection of eight images; z = 0.7 μm. (I, J) Histograms comparing the reduction of rod and cone BCs (per 300-μm lateral distance) in microbead-injected Cx36<sup>++/++</sup> (red) and Cx36<sup>−/−</sup> mice (green) compared with control (black) levels. Data are presented as mean ± SEM. **P < 0.01; n = 5 eyes/group. (K) Histogram quantifies the density of rod BC axon terminals in the IPL of glaucomatous Cx36<sup>++/++</sup> and Cx36<sup>−/−</sup> mice as compared with control eyes. Data are presented as mean ± SEM. **P < 0.01; n = 5 eyes/group. Scale bar: 10 μm in all panels. Conventions are the same as in Figure 1.
remodeled to a punctate shape (Fig. 4E, inset), which were no longer clearly associated with dendritic tips of RBCs. Despite these changes to the photoreceptor synaptic complex, there was no measurable loss of photoreceptors over the 8-week experimental period. We occasionally observed RBC dendrites sprouting (Figs. 4D, 4F), a process...
of remodeling, presumably to make ectopic contact with photoreceptor terminals. In contrast, we observed no significant changes in RBC dendritic structure, nor in the number and shape of the CtBP2-positive synaptic ribbons, in the OPL of microbead-injected \textit{Cx36}\textsuperscript{−/−} mice (Figs. 4G–4J).

We next investigated changes in the distribution of vesicular glutamate transporter 1 (VGLUT1) in glaucomatous eyes, which is responsible for the uploading of glutamate into synaptic vesicles. Consistent with earlier studies, immunostaining of control retinas (Fig. 5A) showed discrete punctate labeling for VGLUT1 throughout the IPL (Fig. 5B). Double-labeling experiments identified large PKCa-positive RBC axon terminals in distal layer 5 of the IPL with VGLUT1 expression (Fig. 5A). In glaucomatous retinas, the density of VGLUT1-positive structures was reduced in the IPL by 34\% ($P < 0.05$; $n = 4$ eyes/group) (Figs. 5C, 5D, 5I). This reduction was evident for VGLUT1-positive patches that were colocalized with the PKCa-positive terminals deep in layer 5 of the IPL (Fig. 5E). The reduced VGLUT1 expression throughout both sublamina in the IPL strongly suggests synaptic changes to CBC axon terminals, as well.

In contrast, we observed no significant change in the pattern of VGLUT1 immunolabeling in microbead-injected \textit{Cx36}\textsuperscript{−/−} mice (Figs. 5E, 5F, 5I). Similarly, pharmacological block of GJs by application of MFA prevented changes in VGLUT1 expression in microbead-injected WT mouse retinas over the 8-week experimental period (Figs. 5G–5I).

**Altered Morphology of Horizontal Cells in Glaucomatous Retinas**

A single type of axon-bearing HCs in mouse makes synaptic contacts with cone and rod photoreceptors via dendritic and axonal endings, respectively. The HCs form an electrically coupled network important to contrast sensitivity, a feature often impaired in glaucoma patients. We therefore looked for morphological changes in HC structure in glaucomatous retinas by labeling vertical sections with anti-calbindin (CB) (Fig. 6A). We found no significant difference between the number of HC somata in control retinas and those in microbead-injected eyes ($P > 0.5$; $n = 3$ eyes/group), but we did see structural changes in dendritic and axonal processes. In addition to the thick proximal dendritic processes, distal punctate terminal endings could be visualized at the level of photoreceptor terminals (arrowheads). However, we found that to see the terminal puncta it was necessary to increase the gain of the photomultiplier of the confocal microscope, which resulted in a saturated image. Although these synaptic terminals have been described previously as dendritic tips, it is possible that some puncta may be axon terminals. Nevertheless, at 8 weeks after microbead injection, we found that CB-positive terminal puncta showed a clear change in structure (Fig. 6B). Although background CB label could be visualized, there was a clear loss of the punctate structure seen in control retinas. In contrast, retinas from microbead-injected eyes of
Outer Retinal Changes in Glaucoma

**FIGURE 6.** Remodeling of horizontal cell dendrites and axon terminals in glaucomatous eyes was prevented by GJ blockade with MFA or ablation of Cx36. (A, B) Retinas from control WT eyes immunolabeled with anti-calbindin (CB) to visualize HC somata and dendrites in vertical sections and anti-SMI32 to visualize HC axon terminals in whole mounts. Arrowheads point to beaded synaptic endings presumably from dendrites. (C, D) At 8 weeks after initial microbead injection, there were structural changes in WT mouse retinas in terms of the visible structure of synaptic endings and the density of axon terminals. (E, F) At 8 weeks after initial microbead injection, the HC dendritic and axon terminal structure in Cx36−/− mouse retinas was comparable to that seen in control eyes. (G, H) Similarly, at 8 weeks after initial microbead injection, WT mouse eyes treated with MFA showed HC structure comparable to that in control eyes. (I) Histogram quantifies changes in the density of HC axon terminals in different mouse strains and protocols. Data in panels I and J are presented as mean ± SEM. *P < 0.01, **P < 0.05; n = 3 eyes/group. Scale bar: 10 μm in the top panels and 20 μm in the bottom panels. Panels A to D are the projection of five images; z = 0.7 μm. Conventions are the same as in Figure 1.

Cx36−/− mice or WT mice treated with MFA maintained the punctate structure of terminal endings seen in control retinas (Figs. 6C, 6D).

The axon terminals of HCs in mouse retina express a non-phosphorylated neurofilament, which can be visualized by anti-SMI32 labeling. The OPL of control retinas contained an extensive meshwork of laterally arborizing processes of HC axon terminals (Fig. 6B). In contrast, the coverage of SMI32-positive axon terminals was reduced by 39% (P < 0.05; n = 3 eyes/group) in glaucomatous retinas with spared processes that were shorter and less ramified than controls (Figs. 6D, 6J). In contrast, retinas from microbead-injected Cx36−/− (Fig. 6F) or MFA-treated WT (Fig. 6H) mice showed no significant changes (P > 0.5; n = 3 eyes/group) in density or appearance of SMI32-positive axon terminals (Fig. 6J).

**DISCUSSION**

Despite findings of abnormalities in the outer retina of human patients and animal models with glaucoma, it has remained unclear whether these changes result directly from glaucomatous insult or are secondary consequences of inner retinal pathology. Our findings support and extend the evidence for outer retinal damage in experimental glaucoma but also provide strong evidence that outer retinal changes occur subsequent to and result as a consequence of inner retinal damage.

Consistent with earlier glaucoma studies, we found that sustained elevation of IOP negatively affected the ERG b-wave in WT mice, suggesting a reduction of ON BC activity. We found a corresponding loss of both RBCs and CBCs in glaucomatous eyes. A loss of RBC immunoreactivity for PKCα has been reported earlier in a vein cauterization model of glaucoma in rats, but it was not clear whether these cells died or had stopped expressing the molecular marker. The decreased number of PKCα-positive RBCs, as well as Chx10-positive CBCs, in our study is most likely attributable to cell death rather than a change in protein expression, as surviving BCs showed intensity of immunolabeling comparable to that in control retinas. Structural changes to dendrites and axon terminals have been reported in a number of ocular pathologies, including glaucoma. Our findings support and extend the
changes described in the DBA/2J mouse model of congenital glaucoma.66

We observed a significant alteration in CtBP2 immunostaining in the OPL in glaucomatous retinas, indicating damage to the photoreceptor synaptic complex. Such changes corresponded to dendritic changes of second-order BCs and HCs, which included retraction of lip processes and occasional abnormal growth. The morphological alterations of structures in the OPL are consistent with the functional impairment indicated by changes in the ERG b-wave. In the IPL, surviving RBC axon terminal endings in glaucomatous retinas retained PKCα expression but lost the VGlut1 immunoreactivity found in control retinas. This suggests that synaptic release of glutamate from BCS to postsynaptic targets is diminished, as well.

The first significant loss of RBCs was first observed 4 weeks after microbead injection, which coincides with the earliest reported loss of RGCs and ACs.28,32 In contrast, significant loss of CBCs was first seen at 5 weeks after microbead injection. Changes in dendritic architecture preceding neuronal loss have been described in animal models of glaucoma.40,41 We found significant loss of dendritic processes in RGCs as early as 3 weeks after the initial microbead injection. However, damage to RBC axon terminals was first observed at 4 weeks, a significant difference in timing relative to our 8-week model.

Overall, these findings support the idea that structural changes to outer retinal neurons occur subsequent to those in the inner retina but do not address whether they result as a consequence. To examine this question, we determined whether neuroprotection of inner retinal neurons could also prevent damage in the outer retina. Indeed, we found that genetic ablation of Cx36 or blockade of GJs with MFA prevented structural damage to BCS and HCs, preserved the ERG b-wave at control levels, and sustained synaptic structures in the OPL and IPL of glaucomatous retinas. Combined with our previous finding that ablation of Cx36-expressing GJs protects RGCs and ACs,28 these results support the idea that outer retina damage is a secondary consequence of inner retinal pathology in glaucoma. Yet, the question may still be raised whether GJ blockade or, in particular, Cx36 does not result from the primary insult but rather from the loss of their postsynaptic target cells, consistent with attenuated VGlut1 expression in BC axon terminals. This mechanism has been linked to cell death in numerous CNS neurological diseases, including recently in glaucoma.27,77–79 Although CBCs synapse directly with degenerating RGCs, BRCs do not; however, BRCs make synaptic contacts with ACs, which have been shown to degenerate in glaucomatous eyes with a time course similar to that for the loss of RGCs.28 In turn, the structural changes in BC dendrites could then lead to the observed alterations in photoreceptor terminal synaptic complexes that, in turn, initiate the postsynaptic degeneration of HC dendritic and axonal processes.

Another potential mechanism for the spread of retinal damage is reactive gliosis, which is often manifested as an increase in glial fibrillary acidic protein expression in astrocytes lying adjacent to the nerve fiber layer and in Müller cell processes that extend vertically through retinal layers.28,80,81 Reactive gliosis can alter environmental homeostasis and release toxins that can lead to progressive neuronal degeneration.82,83 Interestingly, ablation of Cx36 or blockade of GJs, which we show here can prevent outer retinal changes in glaucoma, has been reported to prevent gliosis.28 Although IOP-lowering drugs remain the mainstay treatment for glaucoma, recent studies have turned to neuroprotective strategies to prevent cell death and subsequent vision loss.84 Importantly, therapies providing protection to the inner retina but leaving outer retinal damage unaffected would be pointless, as dysfunctional BC signaling to RGCs would still lead to vision defects. It is therefore fortuitous that our findings show that protection of the inner retina by GJ blockade can also prevent damage to outer retinal neurons, including their synaptic connections. Our findings thus reveal inner retinal neurons as potential targets for effective neuroprotective therapies to sustain vision in glaucoma patients.

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**References**

1. Quigley HA. Neuronal death in glaucoma. *Prog Retin Eye Res.* 1999;18(1):39–57.
2. Schwartz M. Neurodegeneration and neuroprotection in glaucoma: development of a therapeutic neuroprotective vaccine: the Friedenwald lecture. *Invest Ophtalmol Vis Sci.* 2003;44(4):1407–1411.
3. Kendall KR, Quigley HA, Kerrigan LA, Pease ME, Quigley EN. Primary open-angle glaucoma is not associated with photoreceptor loss. *Invest Ophthalrnol Vis Sci.* 1995;36(1):200–205.
4. Frishman LJ, Shen FF, Du L, et al. The scotopic electroretinogram of macaque after retinal ganglion cell loss from experimental glaucoma. *Invest Ophthalmol Vis Sci.* 1996;37(1):125–141.
5. Levkovich-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophtalmol Vis Sci.* 2002;43(2):402–410.
6. Cifuentes-Canorea P, Ruiz-Medrano J, Gutierrez-Bonet R, et al. Analysis of inner and outer retinal layers using
spectral domain optical coherence tomography automated segmentation software in ocular hypertensive and glaucoma patients. *Plos One.* 2018;13(4):e0196112.
7. Nork TM, Ver Hoeve JN, Poulsen GL, et al. Swelling and loss of photoreceptors in chronic human and experimental glaucomas. *Arch Ophthalmol.* 2000;118(2):235–245.
8. Lei Y, Garrahan N, Herrmann B, et al. Quantification of retinal transneuronal degeneration in human glaucoma: a novel multiphoton-DAPI approach. *Invest Ophthalmol Vis Sci.* 2008;49(5):1940–1945.
9. Choi SS, Zawadzki RJ, Lim MC, et al. Evidence of outer retinal changes in glaucoma patients as revealed by ultrahigh-resolution in vivo retinal imaging. *Br J Ophthalmol.* 2011;95(1):131–141.
10. Georgiou AL, Guo L, Francesca Cordeiro M, Salt TE. Electrotetrogram and visual-evoked potential assessment of retinal and central visual function in a rat ocular hypertension model of glaucoma. *Curr Eye Res.* 2014;39(5):472–486.
11. Ortín-Martínez A, Salinas-Navarro M, Nadal-Nicolás FM, et al. Laser-induced ocular hypertension in adult rats does not affect non-RGC neurons in the ganglion cell layer but results in protracted severe loss of cone-photoreceptors. *Exp Eye Res.* 2015;132:17–33.
12. Velten IM, Horn FK, Korth M, Velten K. The b-wave of the dark adapted flash electroretinogram in patients with advanced asymmetrical glaucoma and normal subjects. *Br J Ophthalmol.* 2001;85(4):403–409.
13. Hernandez M, Rodriguez FD, Sharma SC, Vecino E. Immuno-histochemical changes in rat retinas at various time periods of elevated intracocular pressure. *Mol Vis.* 2009;15:2066–2709.
14. Cuenca N, Pinilla I, Fernández-Sánchez L, et al. Changes in the inner and outer retinal layers after acute increase of the intracocular pressure in adult albino Swiss mice. *Exp Eye Res.* 2010;91(2):273–285.
15. Cuenca N, Fernández-Sánchez L, Campello I, et al. Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. *Prog Retin Eye Res.* 2014;43:17–75.
16. Fernández-Sánchez L, de Sevilla Müller LP, Brecha NC, Cuenca N. Loss of outer retinal neurons and circuitry alterations in the DBA/2J mouse. *Invest Ophthalmol Vis Sci.* 2014;55(9):6059–6072.
17. Pang JJ, Frankort BJ, Gross RL, Wu SM. Elevated intracocular pressure decreases response sensitivity of inner retinal neurons in experimental glaucoma mice. *Proc Natl Acad Sci USA.* 2015;112(8):2593–2598.
18. Janssen P, Naskar R, Moore S, Thanos S, Thiel HJ. Evidence for glaucoma-induced horizontal cell alterations in the human retina. *Ger J Ophthalmol.* 1996;5(6):378–385.
19. Euler T, Haverkamp S, Schubert T, Baden T. Retinal bipolar cells: elementary building blocks of vision. *Nat Rev Neurosci.* 2014;15(8):507–519.
20. Chaya T, Matsumoto A, Sugita Y, et al. Versatile functional roles of horizontal cells in the retinal circuit. *Sci Rep.* 2017;7(1):1–15.
21. Ströh S, Puller C, Swirski S, et al. Eliminating gluatamateergic input onto horizontal cells changes the dynamic range and receptive field organization of mouse retinal ganglion cells. *J Neurosci.* 2018;38(8):2015–2028.
22. Kallonitakis M, Harwerth RS, 3rd Smith EL, De Santis L. Colour vision anomalies following experimental glaucoma in monkeys. *Ophthalmic Physiol Opt.* 1993;13(1):56–67.
23. Sabharwal J, Seilheimer RL, Tao X, Cowan CS, Frankort BJ, Wu SM. Elevated IOP alters the space-time profiles in the center and surround of both ON and OFF RGCs in mouse. *Proc Natl Acad Sci USA.* 2017;114(33):8899–8904.
24. Shakarchi AF, Mihailovic A, West SK, Friedman DS, Ramulu PY. Vision parameters most important to functionality in glaucoma. *Invest Ophthalmol Vis Sci.* 2019;60(14):4556–4563.
25. Werner JS, Keltner JL, Zawadzki RJ, Choi SS. Outer retinal abnormalities associated with inner retinal pathology in nonglaucomatous and glaucomatous optic neuropathies. *Eye (Lond).* 2011;25(3):279–289.
26. Calkins DJ. Critical pathogenic events underlying progression of neurodegeneration in glaucoma. *Prog Retin Eye Res.* 2012;31(6):702–719.
27. Lawlor M, Danesh-Meyer H, Levin LA, Davagnanam I, De Vita E, Plant GT. Glaucoma and the brain: trans-synaptic degeneration, structural change, and implications for neuroprotection. *Surv Ophthalmol.* 2018;63(3):296–308.
28. Akopian A, Kumar S, Ramakrishnan H, Roy K, Viswanathan S, Bloomfield SA. Targeting neuronal gap junctions in mouse retina offers neuroprotection in glaucoma. *J Clin Invest.* 2017;127(7):2647–2661.
29. Deans MR, Volgyi B, Goodenough DA, Bloomfield SA, Paul DL. Connexin36 is essential for transmission of rod-mediated visual signals in the mammalian retina. *Neuron.* 2002;36(4):703–712.
30. Sappington RM, Carlson BJ, Crish SD, Calkins DJ. The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest Ophthalmol Vis Sci.* 2010;51(1):207–216.
31. Akopian A, Atlasz T, Pan F, et al. Gap junction-mediated death of retinal neurons is connexin and insult specific: a potential target for neuroprotection. *J Neurosci.* 2014;34(32):10582–10591.
32. Akopian A, Kumar S, H, Viswanathan S, Bloomfield SA. Amacrine cells coupled to ganglion cells via gap junctions are highly vulnerable in glaucomatous mouse retinas. *J Comp Neurol.* 2019;527(1):159–173.
33. Penn RD, Hagins WA. Signal transmission along retinal rods and the origin of the electroretinographic a-wave. *Nature.* 1969;233(5202):201–205.
34. Robson JG, Frishman LJ. Response linearity and kinetics of the cat retina: the bipolar cell component of the dark-adapted electroretinogram. *Vis Neurosci.* 1995;12(5):837–850.
35. Stockton RA, Slaughter MM. B-wave of the electroretinogram. A reflection of ON bipolar cell activity. *J Gen Physiol.* 1989;93(1):101–122.
36. Greferath U, Grünert U, Wässle H. Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. *J Comp Neurol.* 1990;301(3):433–442.
37. Haverkamp S, Wässle H. Immunocytochemical analysis of the mouse retina. *J Comp Neurol.* 2000;424(1):1–23.
38. Morrow EM, Chen CM, Cepko CL. Temporal order of bipolar cell genesis in the neural retina. *Neural Dev.* 2008;3(1):1–9.
39. Burmeister M, Novak J, Liang MY, et al. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat Genet.* 1996;12(4):376–384.
40. Morgan JE, Datta AV, Erichsen JT, Albon J, Boulton ME. Retinal ganglion cell remodelling in experimental glaucoma. *Adv Exp Med Biol.* 2006;572:397–402.
41. El-Danaf RN, Huberman ADJ. Characteristic patterns of dendritic remodelling in early-stage glaucoma: evidence from genetically identified retinal ganglion cell types. *J Neurosci.* 2015;35(6):2329–2343.
42. Spray DC, Hanstein R, Lopez-Quintero SV, Stout RF, Suadciani SO, Thi MM. Gap junctions and bystander effects: Good Samaritans and executioners. *Wiley Interdiscip Rev Membr Transp Signal.* 2013;2(1):1–15.
43. Danesh-Meyer HV, Zhang J, Acosta ML, Rupenthal ID, Green CR. Connexin43 in retinal injury and disease. *Prog Retin Eye Res*. 2016;51:41–68.

44. O’Brien J, Bloomfield SA. Plasticity of retinal gap junctions: role in synaptic physiology and disease. *Annu Rev Vis Sci*. 2018;4:79–100.

45. Dieck S, Altrock WD, Kessels MM, et al. Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. *J Cell Biol*. 2005;168(5):825–836.

46. Cuenca N, Pinilla I, Sauvé Y, Lu B, Wang S, Lund RD. Regressive and reactive changes in the connectivity patterns of rod and cone pathways of P23H transgenic rat retina. *Neuroscience*. 2004;127(2):301–317.

47. Liets LC, Eliasieh K, Van Der List DA, Chalupa LM. Dendrites of rod bipolar cells sprout in normal aging retina. *Proc Natl Acad Sci U S A*. 2006;103(32):12156–12160.

48. Sherry DM, Zhang J, Acosta ML, Rupenthal ID, Green CR. Connexin expression of retinal neurons in a mouse model of retinitis pigmentosa. *J Comp Neurol*. 2005;486(4):480–498.

49. Johnson J, Tian N, Caywood MS, Reimer RJ, Edwards RH, Copenhagen DR. Vesicular neurotransmitter transporter expression in developing postnatal rodent retina: GABA and glycine precede glutamate. *J Neurosci*. 2003;23(2):518–529.

50. Haverkamp S, Ghosh KK, Hirano AA, Wäsche H. Immunochemical detection of five bipolar cell types of the mouse retina. *J Comp Neurol*. 2003;455(4):463–476.

51. Kolb H. The connections between horizontal cells and photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. *J Comp Neurol*. 1974;155(1):1–14.

52. Peichl L, González-Soriano J. Morphological types of horizontal cell in rodent retina: a comparison of rat, mouse, gerbil, and guinea pig. *Vis Neurosci*. 1994;11(3):501–517.

53. Lipin MY, Smith RG, Taylor WR. Maximizing contrast resolution in the outer retina of mammals. *Biol Cybern*. 2010;103(1):57–77.

54. Hawkins AS, Szlyk JP, Ardickas Z, Alexander KR, Wilen- CR. Connexin43 in retinal injury and disease. *Prog Retin Eye Res*. 2016;51:41–68.

55. Hu CX, Zhang J, Brodie S, et al. What do patients with glaucoma see? Visual symptoms reported by patients with glaucoma. *J Glaucoma*. 2003;12(2):134–138.

56. Barhoum R, Martinez-Navarrete G, Corrochano S, et al. Functional and structural modifications during retinal degeneration in the rd10 mouse. *Neuroscience*. 2010;168(3):698–713.

57. Phillips MJ, Otteson DC, Sherry DM. Progression of neuronal and synaptic remodeling in the rd10 mouse model of retinitis pigmentosa. *J Comp Neurol*. 2010;518(11):2071–2089.

58. Fernández-Bueno I, Fernández-Sánchez L, Gayoso MJ, García-Gutiérrez MT, Pastor JC, Cuenca N. Time course modifications in organotypic culture of human neuroretina. *Exp Eye Res*. 2012;104:26–38.

59. Feigenspan A, Janssen-Bienhold U, Corrochano S, et al. Deletion of connexin45 in mouse models of retinitis pigmentosa leads to impaired visual transmission. *J Neurosci*. 2005;25(3):566–5676.

60. Fernández-Bueno JR, Chen M, Penalva RG, Xu H. Loss of synaptic connectivity, particularly in second order neurons is a key feature of diabetic retinal neuropathy in the Ins2Akita mouse. *PLoS One*. 2014;9(5):e97970.

61. Holopigian K, Seiple W, Mayron C, Koty R, Lorenzo M. Immunocytochemical detection of five bipolar cell types of the mouse retina. *J Comp Neurol*. 2003;455(4):463–476.

62. Dedek K, Schultz K, Pieper M, et al. Localization of heterotypic gap junctions composed of connexin45 and connexin36 in the rod pathway of the mouse retina. *Eur J Neurosci*. 2004;19(10):2633–2640.

63. Liets LC, Eliasieh K, Van Der List DA, Chalupa LM. Dendrites of rod bipolar cells sprout in normal aging retina. *Proc Natl Acad Sci U S A*. 2006;103(32):12156–12160.

64. Sherry DM, Zhang J, Acosta ML, Rupenthal ID, Green CR. Connexin expression of retinal neurons in a mouse model of retinitis pigmentosa. *J Comp Neurol*. 2005;455(4):480–498.

65. Holopigian K, Seiple W, Mayron C, Koty R, Lorenzo M. Immunocytochemical detection of five bipolar cell types of the mouse retina. *J Comp Neurol*. 2003;455(4):463–476.

66. Dedek K, Schultz K, Pieper M, et al. Localization of heterotypic gap junctions composed of connexin45 and connexin36 in the rod pathway of the mouse retina. *Eur J Neurosci*. 2006;24(6):1578–1592.

67. Fishman PA, Fishman GC, Fishman LM, et al. Research on photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. *J Comp Neurol*. 1974;155(1):1–14.

68. Dedek K, Schultz K, Pieper M, et al. Localization of heterotypic gap junctions composed of connexin45 and connexin36 in the rod pathway of the mouse retina. *Eur J Neurosci*. 2004;24(13):3325–3334.

69. Fishman PA, Fishman GC, Fishman LM, et al. Research on photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. *J Comp Neurol*. 1974;155(1):1–14.

70. Fishman PA, Fishman GC, Fishman LM, et al. Research on photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. *J Comp Neurol*. 1974;155(1):1–14.
81. Sun D, Moore S, Jakobs TC. Optic nerve astrocyte reactivity protects function in experimental glaucoma and other nerve injuries. *J Exp Med.* 2017;214(5):1411–1430.
82. Bosco A, Breen KT, Anderson SR, Steele MR, Calkins DJ, Vetter ML. Glial coverage in the optic nerve expands in proportion to optic axon loss in chronic mouse glaucoma. *Exp Eye Res.* 2016;150:34–43.
83. Liddelow SA, Marsh SE, Stevens B. Microglia and astrocytes in disease: dynamic duo or partners in crime? *Trends Immunol.* 2020;41(9):820–835.
84. Almasieh M, Levin LA. Neuroprotection in glaucoma: animal models and clinical trials. *Annu Rev Vis Sci.* 2017;3:91–120.