Targeting amyloid- β in glaucoma treatment

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The development of the devastating neurodegenerative condition, Alzheimer's disease, is strongly associated with amyloid- β (A β) deposition, neuronal apoptosis, and cell loss. Here, we provide evidence that implicates these same mechanisms in the retinal disease glaucoma, a major cause of irreversible blindness worldwide, previously associated simply with the effects of intraocular pressure. We show that $A\beta$ colocalizes with apoptotic retinal ganglion cells (RGC) in experimental glaucoma and induces significant RGC apoptosis in vivo in a dose- and time-dependent manner. We demonstrate that targeting different components of the $A\beta$ formation and aggregation pathway can effectively reduce glaucomatous RGC apoptosis in vivo, and finally, that combining treatments (triple therapy) is more effective than monotherapy. Our work suggests that targeting the A β pathway provides a therapeutic avenue in glaucoma management. Furthermore, our work demonstrates that the combination of agents affecting multiple stages in the A β pathway may be the most effective strategy in A β -related diseases.

combination therapy | neuroprotection | retinal ganglion cell apoptosis

lthough glaucoma, a major cause of blindness worldwide (1), A line of the second se the precise means by which IOP may lead to the irreversible destruction of retinal ganglion cells (RGCs, which are the nerve cells that transfer visual information from the eye to the brain) is far from clear. Indeed, interpretation of the mechanism is further complicated by the fact that damage can also occur at low IOP. Thus, for example, recent evidence indicates progressive visual-field loss in patients despite normalization of IOP with pressure-lowering treatment strategies (3, 4), which means that an alternative approach to the treatment of glaucoma is urgently needed. The principal step leading to irreversible loss of vision in glaucoma is RGC apoptosis, and the question is what mechanisms precede this cell death. Raised IOP in experimental glaucoma models can clearly precipitate RGC apoptosis (5-7) whatever the actual intervening steps. However, the presence of progressive glaucomatous damage in patients with normalized IOP has focused a growing body of work on alternative strategies to those regulating IOP and especially approaches targeting the cellular mechanisms leading to apoptosis.

Amyloid- β (A β) is the major constituent of senile plaques in Alzheimer's disease (AD), the formation of which, caused by abnormal processing of amyloid precursor protein (APP), has been involved in AD neuropathology, although the proximate cause of the neurodegeneration responsible for cognitive impairment is not clear (8). A β has recently been reported to be implicated in the development of RGC apoptosis in glaucoma, with evidence of caspase-3-mediated abnormal APP processing and increased expression of A β in RGCs in experimental glaucoma (9) and decreased vitreous A β levels (consistent with retinal A β deposition) in patients with glaucoma (10). Further evidence of a link between glaucoma and AD has emerged from studies showing that patients with AD have RGC loss associated with typical glaucomatous changes, such as optic neuropathy and visual functional impairment (11–14), as is also the case in Parkinson's disease (15). In addition, both diseases are chronic neurodegenerative conditions with a strong age-related incidence (16, 17). This finding is further supported by increasing evidence of similar pathological mechanisms involving $A\beta$ leading to RGC loss as implicated in the brain (16, 18–20).

Here, we provide further strong evidence from an animal model of glaucoma supporting the involvement of $A\beta$ in glaucoma-induced apoptosis of RGCs and show that the use of agents targeting multiple phases of the $A\beta$ pathway raises the possibility of a neuroprotective approach to the treatment of glaucoma. By manipulating the $A\beta$ pathway, we investigated three different approaches to targeting $A\beta$ in experimental glaucoma and their combination effects: (*i*) reduction of $A\beta$ formation by a β -secretase inhibitor; (*ii*) clearance of $A\beta$ deposition by an anti- $A\beta$ antibody ($A\beta$ ab); and (*iii*) inhibition of $A\beta$ aggregation and neurotoxic effects with Congo red (CR). In particular, we show that combined treatment (triple therapy) is more effective than either single- or dual-agent therapy.

Results

We have carried out four groups of experiments to explore the potential role of $A\beta$ in glaucoma. Using an established model of glaucoma in rats, we first explored the way $A\beta$ expression is associated with RGC apoptosis, then we assessed $A\beta$ neurotoxicity on RGC cells *in vivo*, and finally, we explored the effectiveness of single- and combined-agent therapies, respectively, targeting $A\beta$ in reducing RGC apoptosis in this model.

Aβ **Expression and RGC Apoptosis in Experimental Glaucoma.** In the established model of glaucoma that we used (5, 6, 21, 22), chronic ocular hypertension (OHT) is surgically induced in one eye of each animal. In these experiments, we observed that the integral IOP, defined as the cumulative effect of IOP elevation over time, increased with time up to 16 weeks (P < 0.01). We sought to determine whether the apoptotic RGC cells resulting from the elevated IOP were linked to a change in the pattern of Aβ deposition. Histological analysis of the effects on the retina

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Abbreviations: A β , amyloid- β ; A β ab, antibody to A β ; AD, Alzheimer's disease; APP, amyloid precursor protein; β SI, β -secretase inhibitor; CR, Congo red; DARC, detection of apoptosing retinal cells; IOP, intraocular pressure; OHT, ocular hypertension; RGC, retinal ganglion cell.

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Fig. 1. A β and RGC apoptosis. (A–D) Experimental glaucoma model. (*E* and *F*) Control. A β deposition was labeled by A β antibody (A and *E*, red) colocalized with RGC apoptosis labeled by fluorescent-labeled annexin 5 (*B* and *E*, green), from an OHT eye at 2 weeks (A–D). Composite (C and *E*) and transmission (*D* and *F*) images of the retinal cross-section show colocalization to retinal ganglion cell layer of A β deposition and RGC apoptosis only in the OHT eye. (G) APP deposition was found to decrease over time (*P* < 0.01) as assessed by histological grading. (Scale bar: 50 μ m.)

of IOP elevation revealed the colocalization of $A\beta$ with apoptotic RGCs (Fig. 1*A–D*) compared with normal control (Fig. 1 *E* and *F*). Quantitative analysis showed a significant increase of $A\beta$ deposition in the retinal ganglion cell layer (RGCL) in OHT eyes at all of the time points observed, compared with control (P < 0.01), with a peak at 12 weeks (P < 0.01) after raised IOP. On the other hand, the amount of full-length APP expression was significantly decreased in the RGCL in OHT rats compared with control (Fig. 1*G*, P < 0.01). As a precursor protein, APP might be expected to decrease at a rate equal to the increased deposition of $A\beta$. The observations on $A\beta$ provide a clear demonstration of $A\beta$ colocalizing to apoptosing RGCs and suggest the potential involvement of $A\beta$ neurotoxicity in the development of glaucomatous RGC death.

Assessment of Intravitreal A β Neurotoxicity and RGC Apoptosis in Vivo. Because A β deposition was found to be associated with RGC apoptosis in our experimental glaucoma model, we next investigated the effects of exogenous A β peptide on RGC apoptosis in vivo. The A β_{1-40} peptide is known to be neurotoxic in the central nerve system (CNS), and the 25–35 portions (A β_{25-35}) are known to contain the neurotoxic elements of A β_{1-40} (23, 24). Recent studies have suggested that the soluble A β_{1-42} peptide oligomer is the most potent neurotoxic form of A β in CNS-derived neuronal cultures (25), and recent data suggest that it is the nonfibrillar



Fig. 2. Effects of A β on RGC apoptosis *in vivo*. Both A β_{1-42} and A β_{25-35} induced time- (A) and dose- (B) dependent levels of RGC apoptosis *in vivo*. (A) A β_{1-42} appeared more toxic to RGCs than A β_{25-35} at much reduced concentrations (0.55 versus 50 nmol, respectively). (B) RGC apoptosis count was found to increase with increasing doses of A β_{25-35} . *, P < 0.05; **, P < 0.01; ***, P < 0.001.

oligomeric aggregate form of A β that is neurocytotoxic (8). We found that both intravitreal A β_{1-42} and A β_{25-35} induced RGC apoptosis in the retina in a time-dependent manner. The peak of RGC apoptosis occurred at 72 h after treatment compared with control (Fig. 24). We demonstrated significantly more RGC apoptosis with 0.55 nmol of A β oligomer compared with 50 nmol of A β_{25-35} (P < 0.05 or P < 0.01; Fig. 2A), consistent with the previous CNS findings that A β_{1-42} oligomer neurotoxicity is 10-fold greater than the insoluble fibrillar form and 40-fold greater than the unaggregated peptide (25). In addition to time-dependent effects [see also supporting information (SI) Movie 1], we also demonstrated dose-dependent effects (Fig. 2B). A β thus has a potent neurotoxic effect on RGCs, in line with its possible role in the precipitation of RGC loss in glaucoma.

Single-Agent Therapies Targeting $A\beta$ in Glaucoma. The findings that $A\beta$ is elevated in experimental glaucoma and that exogenous $A\beta$ induces RGC apoptosis suggest that $A\beta$ could be a factor mediating the apoptotic changes in RGC cells in our glaucoma model. If so, we hypothesized that interventions that target $A\beta$ production or its site of action might be expected to reduce the levels of RGC apoptosis. We used several different methods for reducing the effectiveness of $A\beta$ (Fig. 3), including a β -secretase inhibitor (β SI), CR and $A\beta$ ab. β -Secretase, a membrane-anchored aspartic protease, is responsible for the initial step of APP cleavage in the amyloidgenic pathway leading to the generation of $A\beta$; β SIs therefore inhibit the production of $A\beta$, with evidence of *in vitro* and *in vivo* efficacy in AD-related models (26, 27). It has been shown that CR completely blocks $A\beta$



Fig. 3. Approaches for targeting A β . APP, a transmembrane protein, has two identified processing pathways: a nonamyloidogenic pathway, where APP is cleaved by α - and γ -secretases producing α -APP and p3, respectively, and an amyloidogenic pathway associated with β - and γ -secretase-mediated cleavage of APP. A β may aggregate, deposit, and form ion channels in cell plasma membrane, leading to neuronal death. The β SI is believed to block A β formation by inhibiting β -secretase activity. The A β ab is not only able to clear preexisting A β but also to block further A β aggregation. CR is thought to block A β aggregation and neurotoxicity by interfering with protein misfolding and preventing ion channel formation. +, blocking effects.

aggregation and toxicity in rat hippocampal neuron cultures (28), and it is believed that its inhibitory effects are the result of an interference with $A\beta$ protein misfolding, fibril formation, and aggregation and possibly an action on channel formation (Fig. 3) (28, 29). A\betaabs are thought to work by not only blocking $A\beta$ aggregation (30) but also by increasing $A\beta$ clearance in AD-related animal models (31).

In control experiments for the use of A β ab, we found no significant difference between the level of RGC apoptosis seen with a saline control compared with the IgG1 (null antibody) at any time point. On the other hand, we did see differences between ABab and control. Detection of apoptosing retinal cells (DARC) real-time images (5) (Fig. 4 A-L) and quantitative analysis (Fig. 5A) showed that RGC apoptosis (white spots) was significantly lower than control (Figs. 4A-C and 5A) with A β ab treatment at all of the time points studied, i.e., 3 (P < 0.01), 8 (P < 0.01), and 16 (P < 0.01) weeks (Figs. 4 *D*–*F* and 5*A*). When we used CR, we only observed a significant reduction in RGC apoptosis at 3 (P < 0.01) weeks (Figs. 4G and 5A). The β SI showed a modest reduction of RGC apoptosis at 3 weeks but did not reach significance compared with control (Figs. 4J and 5A). There were no significant effects with CR or β SI at 8 and 16 weeks compared with control (Figs. 4 H, I, K, and L and 5A).

All three treatments appeared to alter the profile of RGC apoptosis in a temporal manner (Fig. 5B) by delaying the development of peak RGC apoptosis as well as influencing the peak level of RGC apoptosis. Hence, untreated OHT eyes were found to have peak levels of RGC apoptosis at 3 weeks (15%) compared with 8 weeks for β SI, CR, and A β ab (7%, 6%, and 3%), respectively (Fig. 5B). It appears that the main effect of the treatments is to suppress the early peak of RGC apoptosis over the first 3 weeks.

In an attempt to assess whether timing of treatment administration affected the development of RGC apoptosis and its profile, we next assessed a group of animals treated with the A β ab at the time of IOP elevation (0 weeks) as opposed to 2 weeks later (2 weeks). Compared with control and 2 weeks, the 0 weeks group showed a significant reduction in RGC apoptosis at 16 weeks after IOP elevation (Fig. 5*C*, *P* < 0.01). Thus, it is likely that a protective agent given at the time of IOP elevation would be more effective than when given at 2 weeks later. The



Fig. 4. Targeting $A\beta$ in experimental glaucoma. *In vivo* DARC images show the effects of different approaches targeting $A\beta$ on RGC apoptosis in OHT rats. Eyes were assessed at 3 (*A*, *D*, *G*, and *J*), 8 (*B*, *E*, *H*, and 16 (*C*, *F*, *I*, and *L*) weeks after IOP elevation with treatments of $A\beta$ ab (*D*–*F*), CR (*G*–*I*), and β SI (*J*–*L*), respectively, compared with control (IgG1, no antibody; *A*–*C*). The white spots represent apoptotic RGCs labeled by annexin 5.



Fig. 5. Effects of targeting $A\beta$ on RGC apoptosis. (*A*) All treatments reduced levels of RGC apoptosis at 3 weeks. $A\beta$ antibody ($A\beta/Ab$) resulted in a significant reduction of RGC apoptosis at 3, 8, and 16 weeks compared with control. CR showed a significant reduction of RGC apoptosis at 3 but not 8 and 16 weeks. The β SI showed a modest (no significant) decrease of RGC apoptosis at 3 but not 8 and 16 weeks. (*B*) RGC apoptosis as a percentage of total RGC count with time after IOP elevation. All three treatments delayed peak RGC apoptosis from 15% to at least 3%. (*C*) Comparing time of administration in terms of efficacy, OHT animals treated with the $A\beta/Ab$ at the time of IOP elevation (0 weeks) as opposed to 2 weeks later showed a significant reduction in RGC apoptosis at 16 weeks after IOP elevation. **, P < 0.01.

A β -targeting therapies in this work may act directly by reducing the initial injury of RGCs and subsequently decreasing the secondary effects of the primary injured RGCs (32), especially those produced by oxidative stress mechanisms. This theory probably explains the shift in the peak RGC apoptosis time point in all treatment groups from 3 to 8 weeks. It is further supported by our finding that A β ab treatment at the time of IOP elevation (0 weeks) was more effective than when given at 2 weeks. Basically, we propose that A β neurotoxicity may be involved soon after IOP elevation occurs and that inhibiting A β production and aggregation at the early stages of glaucoma may offer maximal protection to RGCs.

Overall, the A β ab appeared the most effective in the prevention of RGC apoptosis compared with CR and the β SI. The application of a single dose of the A β ab delayed peak RGC apoptosis and appeared to have prolonged effects, with a reduction of RGC apoptosis up to 16 weeks. These effects may be related to the A β ab not only clearing preexisting A β deposition but also blocking A β aggregation (Fig. 3) (30). Clinical trials with antibodies to A β have also shown effective clearance of A β plaques, improved cognitive function, and decreased cerebral volume in AD patients (31). In comparison, CR dramatically reduced RGC apoptosis at 3 weeks and resulted in a delayed peak of RGC apoptosis at 8 weeks after raised IOP. However, compared with the A β ab, CR appeared to have a shorter window of protection against RGC apoptosis.

Although the effects of the β SI were not significant, they did appear to delay the peak RGC apoptosis. There are grounds for expecting the β SI to be more effective (27), and our findings may reflect the low dose we used.

Effect of Combining Agents Targeting Aβ in Glaucoma. Because each of the agents used above targets different and multiple stages of the A β pathway, we checked their effectiveness in combination to see how these differences affected the outcome. Using the same OHT model described before, we assessed different combinations of the single agents, with the same doses as in the monotherapy. In addition, a half-dose of Aβab was also used in the triple-therapy combination. In combination, the neuroprotective effect of all three agents (triple-ABab therapy) was significantly improved at 3 weeks after IOP elevation compared with A β ab alone (P < 0.05, Fig. 6 A–G). The triple-A β ab therapy resulted in 84% mean reduction of RGC apoptosis compared with 74% by A β ab alone (Fig. 6G). Compared with β SI alone, dual therapy of β SI combined with A β ab showed a significant protective effect on RGC apoptosis (P < 0.05). All other combination therapies showed a significant reduction of RGC apoptosis compared with control, although the results were not significantly better than with A β ab monotherapy (Fig. 6G). Our results suggest that combination therapy targeted at different points of the A β pathway may provide the most promising approach to prevent glaucomatous RGC apoptosis. Thus, the $\hat{A\beta}ab$ and its use in combination therapy may have great potential in glaucoma treatment.

Discussion

We have shown here that $A\beta$ is strongly implicated in the development of RGC apoptosis in experimental glaucoma. We also demonstrate *in vivo* that $A\beta$ peptide induces significant RGC apoptosis. We provide evidence that targeting $A\beta$ and blocking its effects with combination therapy may represent an effective treatment strategy in glaucoma. Our ability to monitor the effects of $A\beta$ therapy *in vivo* with DARC (5) highlights the potential of this imaging technology in assessing the clinical value of glaucoma treatments.

Non-IOP-lowering treatments have become a key research area in glaucoma because the control of IOP has been shown to be inadequate in the prevention of progressive glaucomatous damage (3, 4). Yet at present, all medical clinical treatments in glaucoma is pressure-lowering, with an estimated cost of \$5 billion per annum in America alone by 2011 (33).

Currently, the most widely advocated neuroprotective agents in RGC degeneration are modifiers of the glutamate pathways because excessive activation of glutamate receptors (such as the



Fig. 6. Effect of combining agents targeting $A\beta$ in glaucoma. *In vivo* DARC images show the effects of triple (*C* and *D*) and dual (*E* and *F*) therapies on prevention of RGC apoptosis at 3 weeks after IOP elevation compared with control (*A*) and $A\beta$ ab monotherapy (*B*). (*G*) Triple therapy (triple $A\beta$ ab) significantly reduced RGC apoptosis compared with $A\beta$ ab alone (*, *P* < 0.05). In fact, the triple therapy resulted in 84% mean reduction of RGC apoptosis compared with 74% by $A\beta$ ab monotherapy. All other combining therapies showed significant reduction of RGC apoptosis compared with $A\beta$ ab monotherapy. For comparison, the dashed line represents the results of our previous study, where we had combined two different glutamate modulators (MK801 and an mGlut agonist) (22) and shown a 60% reduction of RGC apoptosis at 3 weeks after IOP elevation.

NMDA receptor) is strongly implicated in the development of RGC apoptosis and loss in glaucoma (22, 34, 35). Memantine is the only clinically approved neuroprotective NMDA antagonist and is currently in a phase III clinical trial of glaucoma (34), although early results suggest that just as in the CNS (36), its

clinical effectiveness has failed to live up to expectations (Allergan press release, January 2007).

Our work demonstrates a potential neuroprotective strategy for glaucoma. At present in the field of glaucoma, there is a clear lack of therapies that target the actual causative cellular processes of glaucoma. Targeting $A\beta$ specifically in the eye will provide a localized therapy limiting generalized side effects associated with systemic administration. Furthermore, it will provide treatment only to those areas with degenerating activity.

In conclusion, we have shown that $A\beta$ is a likely mediator of pressure-induced RGC death and that neutralizing antibodies to $A\beta$ can significantly delay and attenuate RGC apoptosis in experimental glaucoma. Perhaps the most exciting finding of the work has been that combination therapy, targeting three different aspects of the $A\beta$ pathway, produced the maximal reduction of RGC apoptosis (>80%). These findings suggest that by manipulating the signaling pathways that drive RGC apoptosis, it may be possible to protect RGCs from degeneration and loss in glaucoma. In this context, we have shown that $A\beta$ could be a particularly suitable target for therapeutic intervention in the eye, in an area in clear need of novel and cellular-based neuroprotective strategies.

Materials and Methods

Animal Experiments. All procedures were in accordance with the regulations of U.K. Home Office and the statement of Association for Research in Vision and Ophthalmology for the use of animals in research and were performed under general anesthesia. Adult male Dark Agouti rats (150–200 g) were used in this work. All animals were imaged *in vivo* with fluorescent-labeled annexin 5 with our recently established imaging technique DARC (5, 22).

RGC Identification. For identification of RGCs, rats had RGCs retrogradely labeled by the application of 1,1'-dioctadecy1–3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil; DiIC₁₈) (3) (Molecular Probes, Eugene, OR) to both superior colliculi as described previously (5, 6, 37). Ten days after the Dil labeling, the rats underwent either surgery to elevate IOP or $A\beta$ intravitreal application.

Experimental OHT. Unilateral elevation of IOP was surgically induced in left eyes of 25 rats by injection of hypertonic saline (1.80 M) into episcleral veins (5, 6, 21). Contralateral unoperated eyes served as a control. The IOP of both eyes in each rat was measured at regular intervals with a Tonopen XL, and the integral IOP was calculated (5, 6). Animals were imaged with DARC at 2, 3, 8, 12, and 16 weeks (n = 5 at each time point) and killed for histology immediately after imaging.

A β -induced **RGC** Apoptosis. A β_{25-35} (Sigma–Aldrich, St. Louis, MO) dissolved in sterilized water (12.5–50 nmol) or freshly made A β_{1-42} oligomers (Sigma–Aldrich, 0.55 nmol) (25) were intravitreally injected in Dark Agouti rats (A β_{25-35} , n = 32; A β_{1-42} , n = 20). Animals were imaged with DARC at 2, 6, 24, 48, and 72 h, with at least four animals at each time point being killed for histology soon after imaging. The contralateral eyes were used as controls and injected with vehicle (sterilized water).

Treatments for OHT Rats. OHT rats were treated by different therapies targeting A β . Rats at the time of IOP elevation (0 weeks) were given intravitreal injections (5 μ l total volume) with the following A β targets: monoclonal A β ab IgG1k (0.5 mg/ml, n = 5; Biosource, Camarillo, CA), β SI (10 μ g/ml, Z-VLL-CHO, n = 5; Calbiochem, San Diego, CA), CR (1.46 mg/ml, n = 5; Sigma–Aldrich), and IgG1k-purified protein (null antibody, 0.5 mg/ml, n = 5; AbD serotec, Raleigh, NC) and saline (n = 5) as control. All treated animals were imaged with DARC at baseline

and 3, 8, and 16 weeks and then killed immediately for histology. A further study was performed to investigate the effects of timing administration of A β target on prevention of RGC apoptosis. Briefly, OHT rats were intravitreally injected with A β ab (0.5 mg/ml, 5 μ l) at 0 (n = 5) or 2 (n = 5) weeks after IOP elevation or with null antibody (0.5 mg/ml, 5 μ l) as control. For combination therapy, individual treatments were combined with the same doses as in the monotherapy. In addition, a half-dose of A β ab (1/2A β ab) was also used for triple combination. Five OHT rats were used in the each combination treatment.

Histology. After the animals were killed, their eyes were enucleated and fixed immediately in 4% fresh paraformaldehyde overnight, after which the eyes were dissected at the equator, the lens and vitreous were removed, and whole mount retinas were collected.

Confocal Microscopy. Fluorescent retinas were assessed with a confocal laser scanning microscope (CLSM 510 META; Zeiss, Gottingen, Germany) with LSM software. This process allowed visualizing annexin 5-labeled RGC apoptosis, Dil-labeled RGCs, and Cy3- or Cy5-labeled A β and APP in both retina whole mounts and cross-sections. For the whole-mount retina (magnification, ×16), we assessed 81 adjacent fields (each measuring 0.329 mm²) radiating outward from the optic nerve head in the rat, and accounting for 40% of the whole retina. A retinal montage was then constructed for each whole retina (5, 6, 22, 38).

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 $A\beta$ and APP Localization. For immunohistochemical localization of $A\beta$, paraffin cross-sections were incubated with mouse monoclonal $A\beta$ ab (1:500 in PBS) (39), followed by incubation with Cy3- or Cy5-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA) (6). The same procedure was performed for APP immunolabeling with mouse anti-fulllength APP monoclonal antibody (1:1,000 in PBS; Chemicon, Temecula, CA).

Image Analysis. The number of apoptotic RGCs labeled by annexin 5 and RGCs labeled by DiI was counted manually with MetaMorph software (Universal Imaging Corp., West Chester, PA) by observers masked to treatment protocols. The amount of RGC apoptosis was presented either by density (number per area) or RGC apoptosis as percentage of the total RGC count. A β and APP immunolabeling on paraffin cross-sections was assessed by four independent and masked observers who used a grading system and analysis methods well known to our group (6, 40, 41). (Scale: -4 to +4, where 0 = same as control eye, 1 = 1-25% of control, 2 = 26-50% of control, 3 = 51-75% of control, 4 = >75% of control; prefix + is more than, and prefix - less than). Statistical analysis was performed with Student's *t* test, ANOVA, and Pearson's correlation.

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