Axonal degeneration of retinal ganglion cells (RGCs) causes blindness in glaucoma. Currently, there are no therapies that target axons to prevent them from degenerating. Activation of the BAX protein has been shown to be the determining step in the intrinsic apoptotic pathway that causes RGCs to die in glaucoma. A putative role for BAX in axonal degeneration is less well elucidated. BCLXL (BCL2L1) is the primary antagonist of BAX in RGCs. We developed a mCherry-BCLXL fusion protein, which prevented BAX recruitment and activation and the mitochondrial trauma in tissue culture cells exposed to staurosporine. This fusion protein was then packaged into adeno-associated virus serotype 2, which was used to transduce RGCs after intravitreal injection and force its overexpression. Transduced RGCs express mCherry-BCLXL throughout their somas and axons along the entire optic tract. In a model of acute optic nerve crush, the transgene prevented the recruitment of a GFP-BAX fusion protein to mitochondria and provided long-term somal protection up to 12 weeks post injury. To test the efficacy in glaucoma, DBA/2J mice were transduced at 5 months of age, just prior to the time they begin to exhibit ocular hypertension. Gene therapy with mCherry-BCLXL did not affect the longitudinal history of intraocular pressure elevation compared to naive mice but did robustly attenuate both RGC soma pathology and axonal degeneration in the optic nerve at both 10.5 and 12 months of age. BCLXL gene therapy is a promising candidate for glaucoma therapy.

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INTRODUCTION

In glaucoma, blindness is caused by degeneration of retinal ganglion cells (RGCs), neurons that project axons from the retina to the brain. The most prominent risk factor for glaucoma is elevated intraocular pressure (IOP) [1], and currently, lowering IOP is the only viable treatment for this disease. While this treatment is often able to slow the progression of neurodegeneration [2], there is widespread acknowledgement that a therapeutic that directly targets the RGCs and their axons would significantly augment IOP lowering therapies.

Evidence suggests that RGC axons are injured early in glaucoma and can degenerate independently of the soma [3]. Mechanisms of axon degeneration have been an area of intense study for the past two decades. There is evidence for axon degeneration pathways mediated independently by SARM1 [3–5] and BAX [6–9]. In RGCs, BAX is essential for the execution of the caspase-dependent, intrinsic apoptotic program [6, 10, 11], but its role in glaucomatous axon degeneration is controversial [3, 5].

Bcl2l1 (hereafter designated as BclXL) is an anti-apoptotic member of the Bcl2 gene family, and in central nervous system neurons is the primary antagonist of BAX [12]. BCLXL exists primarily at the mitochondria and inhibits BAX activation by preventing BAX from accumulating on the mitochondrial outer membrane [13].

Overexpression of BCLXL protects neurons from death following trophic factor withdrawal and ischemia–reperfusion [14–16]. In RGCs, increasing the intracellular concentration of BCLXL prevents the degeneration of somas and proximal axon segments after axotomy [17, 18].

The DBA/2J mouse is a widely used model of glaucoma that develops spontaneous, asynchronous elevation of IOP around 6 months of age that persists until the mice are nearly a year old [19–21]. This causes degeneration of RGCs and makes the DBA/2J mouse a useful model for studying glaucomatous neurodegeneration. Experimentally, the ages of 10.5 and 12 months are frequently used for assessing degeneration. Some therapeutic approaches are protective exclusively at 10.5 months of age [6, 22], whereas others are protective at both 10.5 and 12 months of age [23–25]. The differential effect of therapies is evidence that multiple axon degenerative pathways contribute to glaucomatous neurodegeneration. Intriguingly, deletion of the Bax gene conferred protection only at 10.5 months of age but deletion of Bim, a protein that is important for activating BAX in RGCs, protected optic nerves (ONs) at both 10.5 and 12 months of age, suggesting that the Bcl2 family may mediate axon degeneration independent of BAX activity in RGCs [6, 24]. Notably, both complete Bax and Bim deletion also affected...
the elevation of IOP, which may have contributed to their protective effect.

Gene therapy is a clinically relevant therapeutic paradigm for treating retinal disease [26]. The premise of gene therapy is to deliver a therapeutic gene to a susceptible population of cells, generally via a viral vector such as a recombinant adeno-associated virus (AAV). AAVs have low immunogenicity, are replication deficient, and are not known to cause any human disease [27]. In mice, intravitreal delivery of AAV serotype 2 (AAV2) of a sufficient titer can transduce around 85% of RGCs [28].

Gene therapy targeted directly to RGCs to prevent BAX activation in a glaucoma model has never been attempted. This approach is clinically relevant and can be performed in wild-type mice that do not have the developmental abnormalities associated with Bax deletion [29]. A previous study investigated BCLXL gene therapy in a model of ON axotomy and achieved significant albeit transient protection of RGCs [18]. The transient nature of the protection may have been due to loss of expression of the transgene, which was expressed using a neuron-specific promoter. Neuron-specific gene expression is rapidly silenced in RGCs following ON injury [30]. Loss of expression can be circumvented by using the Pgap promoter, whose expression is sustained in damaged RGCs [31].

We hypothesized that AAV2-mediated BCLXL gene therapy would prevent RGC degeneration in the DBA/2J mouse model of glaucoma. Overexpression of mCherry-BCLXL protected both somas and axons from glaucomatous neurodegeneration. This result demonstrates a role for the BCL2 family in glaucomatous RGC somal and axonal degeneration.

METHODS

Cloning mCherry-BclXL

Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) and Oligo(dT)15 Primer (Promega) were used to make cDNA from total RNA isolated from BALB/c mouse brain tissue. Polymerase chain reaction (PCR) was used to amplify the coding region of the BclXL transcript from the cDNA using the following primers 5′-AAATGTCTCAGAGCAACCGGGAGCTG-3′ and 5′-CAGTGTCTGGTCACTTCCGACTGAAGAG-3′. This PCR product was digested with XhoI and HindIII (Promega) and ligated into a pmCherry-C1 plasmid (Clontech, Mountain View, CA), with BclXL placed downstream of mCherry. Sequencing was performed to verify that BclXL had the proper sequence and was in frame with mCherry.

**Fig. 1** mCherry-BCLXL colocalizes with the mitochondria and prevents BAX recruitment in vitro. Representative images of D407 cells expressing mCherry (A–D), mCherry-BCLXL (E–H) along with mitoBFP and GFP-BAX 3.5 h after treatment with 1 µM staurosporine (STS). Note that mitoBFP fluorescence is rapidly lost when the mitochondrial outer membrane becomes permeabilized and the mitochondria become fragmented, which is why B has dramatically reduced fluorescence. Scale bar = 10 µm. Live cell imaging was used to track the localization of GFP-BAX for 3.5 h after STS treatment. The percentage of cells in each condition with punctate localization is quantified in I. N = 163 and 146 cells for the mCherry and mCherry-BCLXL expressing groups, respectively. χ² test was used to assess the significance of the difference in percentage between each group at each time point. P < 0.0005 at 30 min, P < 0.0001 at 120 min, and P < 0.00001 at 210 min.
D407 cells were grown in Dulbecco’s modiﬁed Eagle media (DMEM) (Corning, Corning, NY) containing 3% (V/V) fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1% penicillin – streptomycin (V/V) (Thermo Fisher Scientiﬁc, Waltham, MA). The GFP-BAX and mitoBFP constructs have been previously described [32, 33].

D407 cells were nucleofected using a Lonza 4D nucleofector (Lonza, Basel, Switzerland) and treated 24 h later with 1 µM staurosporine (STS) (Alfa Aesar, Ward Hill, MA). Live cell imaging was performed using an Andor Revolution XD spinning disc confocal microscope (Andor, Belfast, UK) in a 37 °C imaging chamber. Images were taken every 3 min for 3.5 h during the experiment. Image analysis was performed using the Imaris 9.2.1 software (Oxford Instruments, Abingdon, UK). The signiﬁcant differences in the distribution between groups were assessed using χ² tests.

Viral packaging

The mCherry-BclXL construct was digested using Nhel and Apal and ligated into a bridge vector created from AAV-Pgk-Cre (a gift from Patrick Aebischer, Addgene plasmid #24593) where the Cre coding region was replaced with a multiple cloning site containing unique Nhel and Apal sites.

Viral packaging was performed by the University of North Carolina – Chapel Hill, Vector Core. AAV2-Pgk-mCherry-BclXL and AAV2-Pgk-GFP-Bax had titers of 1.7 × 10¹³ and 2.3 × 10¹² viral genomes/mL, respectively.

Mouse housing and ethics

Mice were handled in accordance with the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin – Madison. Mice were kept in microisolator cages on a 12-h light/dark cycle and fed a 4% fat diet. Sample sizes were estimated using power calculations (0.8, 20% effective difference) with historical variance estimates for the type of assay used. All data shown used sample sizes that exceeded the “N” estimated by power calculations. Animals were randomly distributed into treatment groups with effort to balance male to female ratios. For DBA/2J mouse experiments, the male:female ratio is speciﬁcally indicated for each cohort in the ﬁgure legends. Any animals that exhibited distress after inclusion in a cohort were removed from the study.

Optic nerve crush (ONC) and intravitreal injections

For all surgeries, mice were anesthetized with 16 mg/mL ketamine and 1.5 mg/mL xylazine. Eyes were anesthetized with 0.5% proparacaine hydrochloride. Postoperative discomfort was alleviated with 0.03 mg/mL buprenorphine.

Intravitreal injection of virus or 1% cholera toxin subunit B (Alexa-488) (CTB-488; Thermo Fisher) was performed as previously described [30]. For experiments requiring injection of multiple viruses, titers were equalized by mixing the viruses followed by a single injection. Four weeks were
allowed for viral genome replication and transgene expression. ONC was performed as previously described [34] using C57BL/6J mice.

DBA/2J experiments and IOP measurements using rebound tonometry
Ten-week-old DBA/2J mice were bilaterally injected with AAV2-Pgk-mCherry-BCLXL. An additional cohort of 10 uninjected DBA/2J mice was used as a naive control. Each cohort contained 5 male and 5 female mice. IOPs were measured every 2 weeks between 9 and 11 a.m. using a Tonolab rebound tonometer (Icare, Finland) as previously described [35]. Eyes that developed corneal abnormalities were excluded. Longitudinal IOP history was analyzed using Generalized Estimating Equation regression modeling (R, v4.0.5).

For experiments measuring ON degeneration, DBA/2J mice between 4 and 5 or 7 months of age were bilaterally transduced with AAV2-Pgk-mCherry-BclXL. Naive mice received no injection. Each cohort of glaucomatous mice contained at least 49 ONs and 29 retinas. We did not use a control viral injection because other studies have found no effect on IOP progression or protection of RGCs by control AAVs [35, 36]. Because glaucomatous disease progresses asymmetrically in the eyes of DBA/2J mice [21], each eye/retina/ON was considered as an independent variable.

Euthanasia, tissue removal, and fixation
Mice were euthanized with pentobarbital sodium and phenytoin sodium (Virbac, Westlake, TX) followed by cervical dislocation. Eyes were enucleated with a proximal piece of the ON attached and placed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, 100 mM phosphate buffer with 150 mM NaCl, pH 7.4) for 1 h. For ONC experiments, mice were euthanized 1 week after ONC. For glaucoma studies, mice were euthanized at 10.5 or 12 months of age. Young DBA/2J cohorts consisted of mice <4 months of age.

Frozen sectioning and staining of whole-eye sections
After fixation, a brief PBS wash was performed. Samples, including both retinas and ONs, were equilibrated in 30% sucrose in PBS overnight at 4 °C. Samples were then frozen on dry ice in plastic molds using O.C.T. Compound (Scigen, Paramount, CA). In all, 8 µm sections were obtained from the Translational Initiatives in Pathology (TRIP) lab at the University of Wisconsin – Madison.

Immunofluorescence was performed as previously described [30]. Mouse anti-BRN3A monoclonal antibody (MAB1585) (EMD Millipore, Temecula, CA) was used at a concentration of 1:50. Rabbit anti-mCherry polyclonal antibody (ab167453) (Abcam, Cambridge, UK) was used at a concentration of 1:500. The anti-mCherry antibody was only used to enhance the signal of the sections that were used to assess transduction efficiency. All other samples showed endogenous mCherry signal. Rabbit anti-calbindin antibody (SWANT, Marly, Switzerland) was used at a concentration of 1:500. The anti-mCherry antibody was only used to enhance the signal of the sections that were used to assess transduction efficiency. All other samples showed endogenous mCherry signal. Rabbit anti-calbindin antibody (SWANT, Marly, Switzerland) was used at a concentration of 1:500. Goat anti-mouse immunoglobulin G (lgG) fluorescein isothiocyanate conjugated to FITC or Alexa488 and goat anti-rabbit IgG conjugated to Texas Red, Alexa594 or Alexa488 (Jackson Immunoresearch, West Grove, PA) were used at a concentration of 1:1000.

Transduction efficiency was calculated as the percentage of 4,6-diamidino-2-phenylindole (DAPI)-positive nuclei that had mCherry-BCLXL...
labeling in the adjacent cytosolic compartment and the percentage of BRN3A-positive nuclei that had adjacent mCherry-BCLXL labeling. One section from four transduced eyes was counted.

Imaging of the ON tract
Euthanized mice were decapitated and the heads were placed in 4% PFA for 24 h. The skullcap and brain were then dissected away, taking care to leave the optic tract intact. Imaging was performed on a Zeiss Discovery V8 Stereofluorescent microscope (Zeiss, Oberkochen, Germany). Images were taken using the Zen blue software (Zeiss).

Whole mounting of retinas, imaging, and calculation of nuclear densities
Retinas were whole mounted as previously described [30] using VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA). Imaging and quantification of cellular density were performed as previously described [30]. Counted nuclei were restricted to cells with nuclear morphology typical of neurons (round euchromatic appearance with prominent nucleoli). At least 29 retinas were counted for each group. Statistical significance was calculated using a one-sided t test, which assumed equal variance between groups.

Quantitative PCR (qPCR)
Retinas that were used for qPCR were flash frozen in microcentrifuge tubes on dry ice. cDNA was created as previously described [30]. qPCR was performed using an ABI Quant Studio 7 RT PCR machine (Thermo Fisher Scientific). Primers for S16, BclXL, Nefl, Nrn1, Thy1, Sncg, Tubb3, Gfap, Hsp27, and Gap43 have been described previously [37]. Absolute abundances of each transcript were calculated using a standard curve of S16 ribosomal protein mRNA [37]. Four groups of three pooled eyes were used for each experimental cohort. Statistical significance between the transcript abundances of different groups were calculated using a one sided t test assuming equal variance.

ON fixation, sectioning, paraphenylenediamine (PPD) staining, and scoring
ONs were processed for PPD staining as previously described [38]. Sections were imaged on a Zeiss Axioimager Z2 upright microscope.

Fig. 4 mCherry-BCLXL expression prevents BAX translocation and attenuates cell loss after optic nerve crush. **A, B** Panels showing mCherry-BCLXL and GFP-BAX expression, respectively, in a retinal whole mount from a mouse that had undergone ONC to induce BAX activation. The cell expressing mCherry-BCLXL shows diffuse localization of GFP-BAX and the cell that is not expressing mCherry-BCLXL has punctate, translocated GFP-BAX. Scale bar = 10 µm. **C** A quantification of the phenomenon displayed in **A, B**. In this experiment, all mice were transduced with AAV2-Pgk-GFP-BAX. Half the mice also received an injection of AAV2-Pgk-mCherry-BCLXL. The percentage of GFP-BAX-labeled cells with punctate GFP-BAX was quantified 1 week after ONC in untransduced mice and mice that had been bilaterally transduced with AAV2-Pgk-mCherry-BclXL, 1 month before ONC (mean ± standard deviation). Significance was determined using a one-sided t test. In all, 4–7 mice were used per group. **D** Cell loss was determined by quantifying the difference in nuclear density in the RGC layer of retinal wholemounts between the experimental and contralateral eye (mean ± standard deviation). In all, 6–10 mice were used per group. n.s. = not significant. *P < 0.05. **P < 0.01.
using a ×40 magnification oil objective. Images were scored using a semi-quantitative three-score system where each nerve was assigned a score of No or Early (NOE), Moderate (MOD), or Severe (SEV) glaucoma [20]. All ONs were scored by two masked observers. The statistical significance between the distributions of different groups was calculated using \( \chi^2 \) test.

**RESULTS**

**mCherry-BCLXL prevents GFP-BAX recruitment in vitro**

The canonical function of mCherry-BCLXL of preventing BAX from accumulating on the mitochondria was verified in vitro. As expected, mCherry-BCLXL colocalized with a mitochondrially...
localized BFP (Fig. 1E, F, H). The ability of mCherry-BCLXL to prevent GFP-BAX translocation was assessed in cells after STS treatment. Over 95% of cells expressing mCherry and GFP-BAX exhibited GFP-BAX translocation to mitochondria within 3.5 h (Fig. 1A–D, I). Conversely, only 8% of cells expressing mCherry-BCLXL and GFP-BAX exhibited punctate GFP-BAX (Fig. 1E–I).

**AAV2-Pgk-mCherry-BclXL efficiently transduces RGCs**

Next, the transduction efficiency of AAV2-Pgk-mCherry-BclXL (Fig. 2A) was measured. Evaluation of retinal whole mounts 4 weeks after intravitreal injection showed wide-spread expression of the transgene in the ganglion cell layer (Fig. 2B). Histologic examination of transduced retinas showed that AAV2-Pgk-mCherry-BclXL transduced over 50% of the cells in the ganglion cell layer of the retina, which is consistent with the percentage of RGCs in this layer [39]. Colocalization with the RGC marker BRN3A demonstrated that nearly 80% of BRN3A+ cells were transduced (Fig. 2C–G). AAV2 also transduces other retinal cell types [31]. We observed transduction of both horizontal and amacrine cells based on colocalization with an antibody to Calbindin [40] (Fig. S1).

The mCherry-BCLXL fusion protein was also observed throughout the optic tract of transduced eyes, demonstrating its presence in RGC axons (Fig. 3). Unilateral injection of CTB-488, to label axons, in a mouse that was bilaterally injected with AAV2-Pgk-mCherry-BclXL showed colocalization with the
fusion protein, confirming axonal labeling. CTB-488 also showed bilateral fluorescence in the suprachiasmatic nucleus (SCN; Fig. 3C arrows), the only region of the rodent brain that is innervated equally on the ipsilateral and contralateral sides by projections from one ON [41], principally from intrinsically photosensitive RGCs [42]. No mCherry-BCLXL fluorescence was detected in the SCN, suggesting that AAV2-Pgk-mCherry-BclXL did not transduce this population of RGCs. Transgenic mice expressing Thy1-mitoCFP to label RGC mitochondria were also injected. ON sections showed colocalization of mCherry-BCLXL and mitochondria in RGC axons (Fig. 3E–G).

**mCherry-BCLXL prevents cell loss in RGC layer following ONC**

We tested the ability of mCherry-BCLXL to protect RGCs from apoptosis following ONC (Fig. 4). Mice were co-transduced with AAV2s expressing mCherry-BCLXL or GFP-BAX. Prevention of BAX translocation after ONC was assessed by counting the percentage of cells with punctate GFP-BAX in the presence or absence of mCherry-BCLXL (Fig. 4A, B). One week after ONC, mCherry-BCLXL overexpression prevented a significant change in the percentage of cells with punctate GFP-BAX (Fig. 4C).

Next, we tested the ability of mCherry-BCLXL to provide sustained protection to RGCs by examining the pattern of cell loss in the RGC layer after ONC. Both 4 and 12 weeks post-ONC, AAV2-Pgk-mCherry-BclXL transduced retinas had significantly less cell loss than untransduced retinas analyzed 4 weeks post-ONC (Fig. 4D).

**AAV2 transduction does not prevent IOP elevation in DBA/2J mice**

Next, we examined whether AAV2-Pgk-mCherry-BclXL affected the progression of ocular hypertension in DBA/2J mice. Histological examination of transduced cells in the conventional aqueous outflow pathway showed that mCherry-BCLXL was expressed in the non-pigmented epithelial cells of the ciliary body but not in cells of the trabecular meshwork or Schlemm’s canal (Fig. 5A–D). Longitudinal measurements of IOP in AAV2-Pgk-mCherry-BclXL transduced mice, compared to untransduced mice, revealed four individual timepoints with statistically significant differences in IOP between groups (Fig. 5E). Regression modeling failed to detect a difference between the trends of the groups over the entire course of the experiment, indicating that mCherry-BCLXL did not significantly impact longitudinal IOP elevation.

**mCherry-BCLXL prevents RGC degeneration in aged DBA/2J mice**

Next, we tested whether mCherry-BCLXL would prevent RGC degeneration in aged DBA/2J mice. Retinal whole mounts showed robust expression of the transgene in transduced retinas at both 10.5 and 12 months of age (Fig. 52). The protective effect of mCherry-BCLXL was first measured by quantifying changes in the abundance of transcripts selectively expressed in RGCs using qPCR. At 10.5 months of age, BCLXL treated retinas exhibited a nearly 5-fold increase in BclXL transcript abundance (Fig. 6A). These retinas also had significantly higher abundances of the RGC-selective mRNAs Nefl, Nrn1, Thy1, Sncg, and Tubb3 transcripts than
untreated retinas, which showed a nearly uniform decrease in these mRNAs (Fig. 6B–F), indicating that BCLXL treatment preserved RGC-specific gene expression. Both naive and treated retinas exhibited similar increases in Gap43 and Hspa27 transcript abundance relative to young mouse, indicating that BCLXL treatment did not prevent retinal stress (Fig. 6G, H). Interestingly, treated retinas expressed more Gap43 (Fig. 6I), a marker of axon regeneration [43].

BCLXL gene therapy prevented glaucomatous degeneration. BCLXL-treated mice had significantly fewer moderately and severely degenerated ONs compared to naive animals at 10.5 months of age (Fig. 7). BCLXL gene therapy was profoundly protective of axons in the ONs of 12-month-old animals, an age when Bax deficiency was not protective [6]. In fact, the distribution of ON scores for 12-month-old BCLXL-treated mice showed modestly, but significantly, less degeneration than the distribution of scores for 10.5-month-old BCLXL-treated mice. Stratification of data where we had both cell density counts and ON scores for the same eye showed that there was an association with reduced cell density and SEV ON score, while eyes exhibiting MOD ON damage typically showed no significant loss of cell density compared to eyes with NOE damage within a given cohort (Fig. 8). We interpret the presence of SEV damage and cell loss in some mice from treated cohorts as a consequence of less-than-optimal viral transduction, although other factors such as extreme ocular hypertension cannot be ruled out. Additionally, cell density in retinas from eyes with NOE ON damage, in most cohorts of aged mice, still exhibited an overall 10% decline in total cell density when compared with young mice, suggesting age-related effects not affected by the gene therapy.

These data reflect the protective effect of BCLXL therapy if applied prophylactically (4–5 months). To test whether we can achieve similar protection during the period of first onset of elevated IOP, mice were transduced at 7 months and aged to 10.5 months. These mice exhibited a similar level of protection compared to mice transduced at an earlier age (Fig. S3).

DISCUSSION

The mCherry-BCLXL fusion protein prevented BAX translocation both in vitro and in vivo (in RGCs) after exposure to acute apoptotic stimuli. In the DBA/2J mouse model of glaucoma, mCherry-BCLXL conferred extended protection to the retina and the ON that exceeded the reported protective effect of genetic deletion of Bax. Therefore, BCLXL must protect RGCs through more than simple inhibition of BAX.

There are several potential explanations for the additional protection conferred by mCherry-BCLXL. Recent literature has focused on the axodegenerative pathway catalyzed by SARM1 [5, 44, 45]. A combination of the Wild-type allele, which prevents SARM1 activation, and depletion of BAX, via genetic deletion of one Bax allele, appeared to provide a greater protective effect to ONs of DBA/2J mice than either treatment alone [3]. Our results suggest that BCLXL is able to inhibit both the BAX and SARM1 pathways. SARM1 activation is the result of an increased ratio of nicotinamide mononucleotide (NMN) to NAD+ [46]. Future studies should examine whether BCLXL stabilizes the ratio of NMN:NAD+ or acts by some indirect mechanism, such as increasing the resiliency of the mitochondria, consistent with growing evidence that an age-related decline in mitochondrial performance is a contributing factor to glaucomatous neurodegeneration [25, 47]. Interestingly, BCLXL has been shown to improve the metabolic efficiency of mitochondria and increase overall mitochondrial biomass [48–50]. Future studies should explore how BCLXL augments mitochondrial function in RGCs.

A final possibility is that BCLXL helps the RGC maintain axonal transport. Axonal transport disruption is one of the earliest events in glaucoma [51]. The sustained protection in glaucomatous animals by our fusion protein suggests that mCherry-BCLXL was present in axons at high enough concentrations throughout the course of the disease to counteract degenerative signaling. A byproduct of putative augmentation of mitochondrial physiology by BCLXL may be uninterrupted axonal transport.

BCLXL gene therapy yielded better preservation of ONs in 12-month-old DBA/2J mice than in 10.5-month-old mice. Since the 10.5- and 12-month groups were performed sequentially, this variation may be the result of a stochastic batch effect. However, the increased abundance of RGC-specific transcripts and the regeneration marker Gap43 suggests some level of spontaneous regeneration occurring in these mice after ocular hypertension subsides, a process which begins between 11 and 12 months of age in these mice [20]. The effect of BCLXL on the regenerative potential of RGCs is a promising, untested future direction.

These results suggest that BCLXL, gene therapy preserves RGC anatomy and gene expression in a mouse model of glaucoma. To build on these findings, future studies should test BCLXL gene therapy in a larger animal model of glaucoma. Larger animals more accurately reflect the human anatomy and disease and the potential challenges with gene therapy in human retinas [52]. Additionally, our experiments did not examine the potential to preserve RGC function, which should be a priority in a model system better suited to clinical examination. Translational application of BCLXL gene therapy will also require evaluating the safety issues associated with long-term expression of this anti-apoptotic protein, including its possible effects on tumorigenesis [53, 54] and increased susceptibility to viral infections [55].
DATA AVAILABILITY

Raw data files used for this study will promptly be made available upon reasonable request to the authors. The reagent “AAV2-Pkg-mCherry-BCLXL” is the subject of a provisional patent filed by the Wisconsin Alumni Research Foundation (WARF).

REFERENCES

1. Quigley HA. Glaucoma. Lancet. 2011;377:1367–77.

2. Leske MC, Heijl A, Hussain M, Bengtsson B, Hyman L, Kometarof E, et al. Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. Arch Ophthalmol. 2003;121:48–56.

3. Howell GR, Libby RT, Jakobs TC, Smith RS, Phalan FC, Barter JW, et al. Axons of retinal ganglion cells are insulated in the optic nerve early in DBA/2J glaucoma. J Cell Biol. 2007;179:1523–37.

4. Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, et al. dsArm1 is required for activation of an injury-induced axon death pathway. Science. 2012;337:481–4.

5. Fernandes KA, Mitchell KL, Patel A, Marola OJ, Shrager P, Zack DJ, et al. Role of SARM1 and DR6 in retinal ganglion cell axonal and somal degeneration following axonal injury. Exp Eye Res. 2018;171:54–61.

6. Libby RT, Li Y, Savinova OV, Barter J, Smith RS, Nickells RW, et al. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. PLoS Genet. 2005. https://doi.org/10.1371/journal.pgen.0010004.

7. Nikolaev A, McLaughlin T, O’Leary DDM, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct cascades. Nature. 2009;457:981–9.

8. Simon DJ, Weiner RM, McLaughlin T, Kalop D, Stanger K, Yang J, et al. A caspase cascade regulating developmental axon degeneration. J Neurosci. 2012;32:17540–53.

9. Simon DJ, Pitts J, Hertz NT, Tesci Mark M, Molina H, Tressier M, et al. Axon degeneration gated by retrograde activation of somatic pro-apoptotic signaling. Cell. 2016;164:1031–45.

10. Li Y, Schlamp CL, Poulsen KP, Nickells RW. Bax-dependent and independent axon pathways of retinal ganglion cell death induced by different damaging stimuli. Exp Eye Res. 2000;70:209–13.

11. Sun YF, Lu LY, Saarma M, Timmusk T, Arumae U. Neuron-specific Bcl-2 homology 3 domain-only splice variant of Bak is anti-apoptotic in neurons, but pro-apoptotic in non-neuronal cells. J Biol Chem. 2001;276:16240–7.

12. Levin LA, Schlamp CL, Spiedloch RG, Geszvain KM, Nickells RW. Identification of the bcl-2 family of genes in the rat retina. Investig Ophthalmol Vis Sci. 1997;38:2545–53.

13. Edlich F, Banerjee S, Suzuki M, Cleland MM, Amourn D, Wang C, et al. Axon degeneration gated by retrograde activation of somatic pro-apoptotic signaling.

14. González-García M, García I, Ding L, O’Connor-O’Leary J, Ong JR, Donahue RJ. Inherited glaucoma in DBA/2J mice: pertinent disease features for studying the mitochondrial energetics by stabilizing the inner membrane potential. J Cell Biol. 2010;180:753–63.

15. Parsadanian AS, Cheng Y, Keller-Peck CR, Holtzman DM, Snider WD. Bcl-x(L) is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc Natl Acad Sci USA. 1995;92:4034–8.

16. Pasadanain AS, Cheng Y, Keller-Peck CR, Holtzman DM, Snider WD. Bcl-x(L) is an anti-apoptotic regulator for postnatal CNS neurons. J Neurosci. 1998;18:1009–19.

17. Yin W, Cao G, Johnnides MJ, Signore AP, Luo Y, Hickey RW, et al. TAT-mediated delivery of a Bcl-XL protein is neuroprotective against neuronal hypoxic-ischemic brain injury via inhibition of caspases and AIF. Neurobiol Dis. 2006;21:358–71.

18. Liu XH, Collier RJ, Youle RJ. Inhibition of axotomy-induced neuronal apoptosis by extracellular delivery of a Bcl-XL fusion protein. J Biol Chem. 2001;276:46326–32.

19. Malik JMI, Shevtsova Z, Bähr M, Kügler S, Shevstova Z, Bahr M, et al. Long-term in vivo inhibition of CNS neurodegeneration by Bcl-XL gene transfer. Mol Ther. 2005;11:73–81.

20. John SWM, Smith RS, Savinova OV, Hawes NL, Chang B, Tumbull D, et al. Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice. Invest Ophthalmol Vis Sci. 1998;39:951–62.

21. Libby RT, Anderson MG, Pang H, Robinson ZH, Savinova OV, Cosma IM, et al. Inherited glaucoma in DBA/2J mice: pertinent disease features for studying the neurodegeneration. Vis Neurosci. 2005;22:1329–41.

22. Schlamp CL, Li Y, Dietz JA, Jansen KT, Nickells RW. Progressive ganglion cell loss and optic nerve degeneration in DBA/2J mice is variable and asymmetric. BMC Neurosci. 2006;7:1–14.

23. Harder JM, Williams PA, Braine C, Yang H, Thomas J, Foxworth NE, et al. Macrophage receptor CSF1R promotes optic nerve degeneration in DBA/2J mice. J Neuroinflammation. 2020. https://doi.org/10.1186/s12974-019-1547-x.

24. Howell GR, Macalino DG, Sousa GL, Walden M, Soto L, Kneeland SC, et al. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. J Clin Invest. 2011;121:1429–44.

25. Harder JM, Fernandes KA, Libby RT. The Bcl-2 family member BM1 has multiple glaucoma-relevant functions in DBA/2J mice. Sci Rep. 2012;2:530.
54. Pena JC, Rudin CM, Thompson CB. A Bcl-x(L) transgene promotes malignant conversion of chemically initiated skin papillomas. Cancer Res. 1998;58:2111–6.

55. Wyzewski Z, Svitlik W, Mielcarska MB, Gregorczyk-Zboroch KP. The role of Bcl-xL protein in viral infections. Int J Mol Sci. 2021;22:1–16.

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AUTHOR CONTRIBUTIONS
RJD and RWN conceived of the project. RJD performed experiments for every portion of the manuscript. RLF performed the in vitro validation of mCherry-BCLXL. JRG performed the analyses for the optic nerve crush experiments. RJD and RWN wrote the manuscript, which was approved by all the authors.

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COMPETING INTERESTS
The Wisconsin Alumni Research Foundation (WABF) has filed a provisional patent application around this research.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
No human subjects were used in this study.

ADDITIONAL INFORMATION
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