Involvement of EphB/Ephrin-B Signaling in Axonal Survival in Mouse Experimental Glaucoma

Christine T. Fu^{1,2,3} and David Sretavan^{1,2,3}

PURPOSE. To examine the functional significance of EphB/ephrin-B upregulation in mouse experimental glaucoma.

METHODS. In a loss-of-function approach, mouse mutants lacking EphB2 (*EphB2*^{-/-}) or EphB3 (*EphB3*^{-/-}) protein, and mutants expressing EphB2 truncated in the C-terminus (*EphB2*^{lacZ/lacZ}) were subjected to laser-induced ocular hypertension (LIOH), an experimental mouse model of glaucoma. The number of optic nerve axons was counted in paraffin-phenylenediamine (PPD)-stained sections and compared between EphB mutants and wild type littermates. In a gain-of-function approach, retina/optic nerve explants obtained from LIOH-treated animals were exposed to EphB2-Fc recombinant proteins or Fc control proteins. Tissue sections through the optic nerve head (ONH) were labeled with neuron-specific anti-tubulin β -III antibody to determine axonal integrity.

RESULTS. Both EphB2 and EphB3 null mutant mice exhibited more severe axonal degeneration than wild type littermates after treatment with LIOH. Mutant mice in which the C-terminal portion of EphB2 is truncated had an intermediate phenotype. Application of EphB2-Fc recombinant protein to LIOH-treated optic nerve explants resulted in greater sparing of tubulin β -III-containing retinal ganglion cell (RGC) axons.

CONCLUSIONS. These results provide genetic evidence in mice that both EphB/ephrin-B forward and reverse signaling feed into an endogenous pathway to moderate the effects of glaucomatous insult on RGC axons. LIOH-induced axon loss is maintained in retina/optic nerve explants after removal from an ocular hypertensive environment. Exogenous application of EphB2 protein enhances RGC axon survival in explants, suggesting that modulation of Eph/ephrin signaling may be of therapeutic interest. (*Invest Ophthalmol Vis Sci.* 2012;53:76–84) DOI:10.1167/iovs.11-8546

As glaucoma is a leading cause of blindness worldwide, the underlying disease mechanisms that impact visual function are of substantial interest. From a consideration of the early patterns of visual field loss in patients, the optic nerve head (ONH) is generally regarded as an important site of pathogenesis resulting in retinal ganglion cell (RGC) axon injury. Support for this view also comes from experimental findings of

significant structural remodeling^{1–7} and obstruction of axoplasmic transport^{4,8–11} at the ONH early in the course of disease. In addition, genetic evidence indicates that when RGC soma apoptosis is prevented, intraretinal axons are largely preserved until they reach the ONH, where massive degeneration ensues.¹² Efforts have been undertaken to identify genes differentially expressed at the glaucomatous ONH to shed some light on the pathways involved in axon injury and survival.^{13–17}

In this study, we focused on the potential role of the Eph/ephrin family of cell surface signaling molecules in optic axon degeneration after glaucomatous injury. The Eph receptor tyrosine kinases and their ephrin ligands function not only in developmental axonal guidance, cell migration, and morphogenesis, but also in adult synaptic plasticity, homeostasis, and cancer.^{18–22} In addition, altered Eph and ephrin expression has been reported in many central nervous system pathologies^{23–25} and genetic studies have demonstrated a functional role for Eph/ephrin signaling in modulating axon survival and regrowth after spinal cord²⁶ and optic nerve injury.²⁷ A key feature of the Eph/ephrin system is that signal transduction occurs bidirectionally^{22,28} and involves both forward and reverse signaling. In forward signaling, ephrin binding to Eph molecules triggers autophosphorylation of the Eph tyrosine kinase domain and a subsequent signaling cascade. In reverse signaling, the binding of an Eph with an ephrin molecule activates signaling pathways within the ephrin-expressing cell.

Among glaucoma-related changes at the ONH, a finding that is consistently observed across multiple animal models and in glaucoma patients is the upregulation of *EphB/ephrin-B* gene expression and protein signaling. The expression of a number of Eph and ephrin family members is upregulated in cultured ONH astrocytes derived from human patients,^{13,29} and in several different animal models of glaucoma including monkey,²⁹ the DBA/2J mouse model of pigmentary glaucoma,^{17,30} and the laser-induced ocular hypertension (LIOH) model in CD-1 mice.³¹ In mice, where this process has been examined in the greatest detail, *EphB/ephrin-B* upregulation is tightly correlated with axon loss,³⁰ and occurs early in disease, preceding or coinciding with the initial morphologic signs of axon damage.³¹ This upregulation of *EphB/ephrin-B* gene expression has been found to be associated with increased active protein signaling in both axons and glia at the ONH.³¹ Furthermore, morphologically normal axons exhibit higher levels of ephrin-B reverse signaling, whereas this signaling pathway is downregulated in aberrant axons.³¹ Despite these correlational findings, whether Eph-ephrin signaling plays a functional role in disease remains unknown.

In the present study, we subjected mouse mutants lacking EphB2 (*EphB2*^{-/-}) or EphB3 (*EphB3*^{-/-}) protein or mutants with engineered alleles of *EphB2* (*EphB2*^{lacZ/lacZ}) to glaucomatous optic nerve damage induced by LIOH. *EphB2* and *EphB3* were chosen as the genes of interest because their mRNAs were shown to be upregulated at the ONH as early as 1 to 2 days after LIOH treatment.³¹ As substantial data indicate axon dysfunction and degeneration precede retinal ganglion

From the Departments of ¹Ophthalmology and ²Physiology, and ³Neuroscience Graduate Program, University of California, San Francisco, San Francisco, California.

Supported by NIH Grant awards EY010688 and EY02162, by a core grant to the UCSF Department of Ophthalmology from Research To Prevent Blindness, and by That Man May See through a gift from Robert Drabkin. D.S. is the recipient of a Senior Scientific Investigator Award from Research To Prevent Blindness.

Submitted for publication September 6, 2011; revised October 28, 2011; accepted October 29, 2011.

Disclosure: C.T. Fu, None; D. Sretavan, None

Corresponding author: David Sretavan, Department of Ophthalmology, University of California, San Francisco, Box 0730, 10 Koret Way, San Francisco, CA 94143; sretavan@vision.ucsf.edu.

FIGURE 1. EphB mutants were comparable with wild type littermates in baseline IOP and IOP elevation after LIOH. IOP measurements from EphB mutants and wild type littermates at baseline before the induction of LIOH (untreated) and 1 day after LIOH treatment (LIOH). No difference in IOP was observed in the baseline IOP and in IOP elevation after LIOH between wild type littermates and EphB mutants. Numbers above the bars represent the sample size of each experimental group. The same cohorts of animals were analyzed for optic nerve axon damage and the results are presented in Figures 2 and 3.

cell body loss,^{32–34} we focused our analysis on the integrity of axons in the optic nerve. Mice totally deficient in EphB2 or EphB3 both exhibited more severe axon degeneration compared with wild type littermates, suggesting that the EphB/ephrin-B pathway normally operates to moderate axon loss in LIOH-induced experimental glaucoma. Exogenous application

of EphB2 recombinant protein attenuated axon degeneration in LIOH-treated optic nerve explants, further supporting the involvement of EphB/ephrin-B signaling in glaucomatous optic nerve pathophysiology.

MATERIALS AND METHODS

Animals

The generation of *EphB2*^{-/-},³⁵ *EphB2*^{lacZ/lacZ},³⁵ and *EphB3*^{-/-}³⁶ mice has been described previously. Mutant mice were generous gifts from Mark Henkemeyer (University of Texas, Southwestern) and were maintained in a CD-1 background as colonies at University of California, San Francisco (UCSF). Homozygous mutant animals were compared with wild type littermates from heterozygous matings for the data shown in Figures 1–4. CD-1 mice used in the optic nerve explant experiments (Fig. 5) were purchased from Charles River Laboratories (Wilmington, MA).

All experiments were performed under protocols approved by the UCSF Institutional Animal Care and Use Committee, and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

LIOH

LIOH was performed in CD-1 mice using previously published procedures.³⁴ In most studies, one eye was treated and the contralateral eye served as untreated control. In optic nerve explant experiments, LIOH was performed in both eyes to compare the effect of EphB2-Fc versus Fc application. IOP was measured with a rebound tonometer (Tonolab; Colonial Medical Supply, Franconia, NH) using procedures described previously.³⁴ Only eyes that exhibited IOP elevation greater than 21 mm Hg after LIOH were used for subsequent experiments.

Paraphenylenediamine (PPD) Staining and Axon Counting

The counting of PPD-stained optic nerve axons has been used extensively to analyze axonal degeneration in the glaucomatous optic nerve.^{37–39} Mice were perfused with 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde fixative. PPD staining and axon counting were performed on optic nerve cross-sections as previously described.³⁴ Briefly, optic nerve samples were dissected from 1 mm behind the globe, postfixed with 1% OsO₄, dehydrated in a graded ethanol series, and embedded in resin. Sections (1 μm thick) were cut and stained

FIGURE 2. EphB mutants exhibited more severe axonal degeneration in experimental glaucoma. (A–H) Representative paraphenylenediamine (PPD)-stained optic nerve cross-sections used for analysis. (A) Wild type littermates, untreated. (B) *EphB2*^{-/-}, untreated. (C) *EphB2*^{lacZ/lacZ}, untreated. (D) *EphB3*^{-/-}, untreated. (E) Wild type littermates, 1 week post-LIOH. (F) *EphB2*^{-/-}, LIOH. (G) *EphB2*^{lacZ/lacZ}, LIOH. (H) *EphB3*^{-/-}, LIOH. Both *EphB2*^{-/-} and *EphB3*^{-/-} mutants showed more axonal degeneration compared with wild type littermates. The *EphB2*^{lacZ/lacZ} line was also more affected than wild type littermates, although its axonal degeneration was not as severe as that in *EphB2*^{-/-}. Scale bar, 10 μm.

FIGURE 3. Quantitative analysis of axon counts in EphB mutants and wild type littermates after LIOH. LIOH treatment in EphB mutants resulted in significant axon loss compared with LIOH in genotype-matched littermates. Both *EphB2*^{-/-} and *EphB3*^{-/-} mutants displayed greater reduction of surviving axon number compared with wild type littermates. Axon degeneration in *EphB2*^{lacZ/lacZ} mice was more severe than wild type, although less so compared with *EphB2*^{-/-}. The number of animals in each experimental group is indicated above the bar graph. In this graph, data from wild type littermates of the three genotypes are represented together in the same column in the untreated condition or after LIOH. **P* < 0.05; ****P* < 0.001.

with 1% PPD in one part methanol/one part isopropanol. Quantification was carried out by a single operator (CF) in a blinded manner. Nonoverlapping image fields spanning the entire optic nerve cross-section were captured using a $\times 100$ objective lens on a microscope (Nikon TE300; Nikon, Melville, NY). The number of healthy myelinated axons was counted manually within randomly sampled $20 \times 15 \mu\text{m}$ fields, and used to calculate the axon density. The density was then averaged and multiplied by the cross-sectional area to obtain the estimated total count of myelinated axons. The criterion for healthy axons was the appearance of dark rings of myelin surrounding unstained axoplasm. Degenerating axons appeared as homogeneously dark and circular profiles.^{38,40} Although this method does not differentiate between intact axons and axons that have undergone molecular alterations but remained structurally normal, it clearly identifies the loss of myelinated axons, which serves as a well-established measure of glaucoma progression.

We primarily used total axon number instead of density to monitor experimental glaucoma progression, due to the potential concern that nerve shrinkage may offset the effect of degeneration on axon density. Our results indicated that the optic nerve size was comparable between EphB mutants and wild type littermates, except for a small reduction in size in *EphB3*^{-/-} animals after LIOH. Analysis using axon density data gave rise to similar results as those presented here.

Cryostat Section Preparation

Mice were anesthetized with an overdose of pentobarbital and perfused transcardially with 4% PFA in 0.1 M phosphate buffered saline (PBS, pH 7.4). Enucleated eyes with a segment of retrobulbar optic

nerve attached were harvested, and the anterior segment, lens, and vitreous were removed. The eye cups were immersion-fixed in the same fixative at room temperature for 2 hours, cryoprotected with 30% sucrose in PBS, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA). Tissues were sectioned at $12 \mu\text{m}$ thickness along the longitudinal axis through the ONH, and mounted on slides (Super Frost Plus; Fisher Scientific, Springfield, NJ).

Immunohistochemistry

Cryosections were washed in 0.1 M PBS and blocked with 10% normal donkey serum (NDS) for 1 hour at room temperature. Triton X-100 (0.1%) was included in the blocking solution to achieve tissue permeabilization. Primary antibodies were incubated overnight at 4°C , followed by PBS washes and 1 hour of secondary antibody incubation at room temperature. Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

The following primary antibodies were used: mouse anti-tubulin β -III (TUJ1; 1:500; Covance Research Products, Denver, PA), and mouse anti-gial fibrillary acidic protein (GFAP; 1:400; Sigma, St. Louis, MO). Colabeling experiments were performed with secondary antibodies conjugated to cy3 and cy5 (1:200; Jackson ImmunoResearch, West Grove, PA) to ensure maximal spectral separation. Confocal images were acquired on a microscope (LSM5 Pascal; Carl Zeiss Meditec, Inc., Thornwood, NY), and pseudocolored in red and green to aid visualization.

Organotypic Culture of Optic Nerve Explants

Optic nerve explants were cultured with retinas attached at the interface of media and a 5% CO_2 /air mixture. RGC axons approximately 5 mm long were in continuity with their cell bodies, prolonging survival in vitro. In addition, the cellular architecture and potential interactions within the optic nerve were preserved by this approach, potentially providing a more physiologically-relevant environment compared with dissociated cell culture. Mouse eyes with connected optic nerves were quickly removed, and the anterior segment, sclera, and meninges were dissected away in cold HEPES-buffered aCSF. Complete removal of meninges around the ONH required great care so as not to damage the nerve. The retina/optic nerve explants were rinsed briefly with pre-equilibrated growth media (containing Neurobasal-A supplemented with B-27 and Penicillin-Streptomycin; Invitrogen, Carlsbad, CA), and transferred onto an organotypic culture insert (Millicell; Millipore, Bedford, MA). One primary cut was made from the retinal periphery to the optic disc region, and the retina (ganglion cell layer facing up) was spread out flat on the organotypic culture insert (Millicell; Millipore) with a custom-made ball-headed instrument. To ensure sufficient gas exchange, this tissue was arranged so that the optic nerve was not covered by the retina, while maintained in a moist condition during the entire procedure. After removal of excessive growth media, the insert was placed into a 6-well plate filled with media. Cultures were inspected every day to make sure a thin film of media remained over the explant, and media change was performed every 2 days.

To compare axonal degeneration between LIOH-treated optic nerves and control unoperated optic nerves in vitro, CD-1 mice (Charles River) were subjected to LIOH in one eye, while the contralateral eye was left untouched. Two days after treatment, optic nerves were harvested and cultured as described above for an additional 4 days. The explants were then fixed in 4% PFA and embedded in OCT. The frozen tissues were cut into $12 \mu\text{m}$ longitudinal sections, and every third section was collected onto the same slide. Only sections through the ONH were analyzed, yielding five to six samples per slide. Neuron-specific anti-tubulin β -III antibody^{41,42} was used to stain the optic nerve sections as a measure of axonal integrity.

To examine the effect of exogenous EphB2 application, we used a recombinant protein of mouse EphB2 fused with a human Fc tag (EphB2-Fc). The Fc tag allows for the purification, detection, and preclustering of EphB2 protein. Functional Eph-ephrin signaling re-

FIGURE 4. Exogenous EphB2-Fc application attenuated axonal degeneration in glaucomatous optic nerve explants. (A–C) Wild type CD-1 mice ($n = 6$) were subjected to LIOH unilaterally. Two days after treatment, both the LIOH-treated and the contralateral control optic nerves were harvested along with the retinas attached and cultured as explants. After 4 days in vitro, the explants were cryosectioned and stained with anti-tubulin β -III antibodies to label axons. Tubulin β -III immunoreactivity remained relatively robust under the culture condition in control optic nerves (A). In LIOH-treated optic nerves (B), tubulin β -III staining was severely diminished, and the labeled axonal profiles demonstrated swellings and undulating trajectories. (C) Quantitative analysis showed that tubulin β -III-positive immunoreactivity was significantly reduced by 50% with LIOH treatment. (D–F) Wild type CD-1 mice ($n = 6$) were subjected to LIOH bilaterally. Optic nerves were cultured as explants 2 days after induction of LIOH. For each animal, one explant was treated with clustered EphB2-Fc recombinant protein, while the explant from the fellow optic nerve received clustered Fc as control. EphB2-Fc application (E) enhanced the preservation of tubulin β -III-positive axons in LIOH-treated explants compared with Fc control (D). The level of immunoreactivity was significantly improved by 80% in the EphB2-Fc treatment group (F). In both (C) and (F), data were analyzed in pairs, with results from each experimental eye normalized against the contralateral control eye. Normalization allowed replicates performed on different days to be compared. ** $P < 0.01$; *** $P < 0.001$.

quires ligand-induced receptor clustering. Soluble ephrin- or Eph-Fc fusion proteins therefore need to be preclustered with anti-Fc antibodies to induce receptor autophosphorylation and activation, while non-clustered fusion proteins may act as antagonists. CD-1 mice were treated with LIOH bilaterally. Two days after treatment, the pair of optic nerves from each mouse was cultured in growth media supplemented with EphB2-Fc (10 μ g/mL; R&D Systems, Minneapolis, MN) or control Fc (10 μ g/mL; Jackson ImmunoResearch) respectively. EphB2-Fc and Fc recombinant proteins were both preclustered with goat anti-human Fc antibodies (20 μ g/mL; Jackson ImmunoResearch) before their addition to the media. Explants were harvested and analyzed after 4 days in vitro.

To demonstrate EphB2-Fc protein binding within optic nerve explants, we cultured non-LIOH treated nerves for 1 day in the presence of Fc or EphB2-Fc preclustered with goat anti-human Fc antibodies. Cryostat tissue sections of the optic nerves from these explants were labeled with additional anti-Fc antibodies to boost the signal, followed by donkey anti-goat secondary antibodies.

Confocal images were analyzed with software developed by Wayne Rasband (ImageJ; National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Thresholds for tubulin fluorescence were set using identical parameters for each pair of optic nerves from the same animal. A 150 $\times$ 200 μ m box was selected at the ONH from each section, in which the extent (pixel area) of the tubulin signal was determined and averaged as a measure of axon survival. Data from the treatment groups were normalized against the contralateral control sample from the same animal.

Statistical Analysis

The EphB mutant IOP and axon quantification data were analyzed by two-way analysis of variance (ANOVA), followed by post hoc Bonferroni multiple comparison test. The optic nerve explant experiments were evaluated with paired *t*-test. Statistical analysis was performed using statistical software (GraphPad Prism, version 5; GraphPad Soft-

ware, La Jolla, CA), with a significance level set at $P < 0.05$. Values were reported as mean $\pm$ SEM.

RESULTS

EphB2 and EphB3 Deficiencies Independently Result in Increased Pressure-Related Axon Degeneration In Vivo

To investigate the potential functional significance of EphB/ephrin-B signaling, we performed LIOH in *EphB2*^{-/-35} and *EphB3*^{-/-36} protein-null mice maintained in the CD-1 background.

Adult *EphB2*^{-/-35} and *EphB3*^{-/-36} mice showed similar intraocular pressures compared with wild type littermates (Fig. 1). The IOP measurements in mm Hg were 13.0 $\pm$ 0.4 for wild type littermates, 12.4 $\pm$ 1.2 for *EphB2*^{-/-}, and 13.0 $\pm$ 1.1 for *EphB3*^{-/-} animals before induction of LIOH. No ocular structural abnormalities were noted by visual inspection using slit-lamp examination. LIOH raised the average IOP in wild type littermates, *EphB2*^{-/-}, and *EphB3*^{-/-} animals to the comparable values of 30.8 $\pm$ 2.8 mm Hg, 30.4 $\pm$ 1.7 mm Hg, and 29.8 $\pm$ 1.8 mm Hg, respectively (measured 1 day postinduction).

Cross-sections of the optic nerves were obtained from *EphB2*^{-/-35}, *EphB3*^{-/-36} mice, and wild type littermates 1 week after LIOH treatment, at which time wild type CD-1 mice have previously been demonstrated to exhibit significant axonal degeneration.³⁴ Optic nerve sections were subjected to PPD staining and analyzed for the number of morphologically intact optic nerve axons.

In wild type littermates not treated with LIOH, PPD labeled healthy myelin sheaths uniformly distributed in optic nerve cross-sections (Fig. 2A). Untreated *EphB2*^{-/-} and *EphB3*^{-/-}

FIGURE 5. Demonstration of EphB2-Fc binding to axons and astrocytes in optic nerve explants. Nerves were harvested from non-LIOH treated animals ($n = 3$) and cultured for 1 day with either Fc or EphB2-Fc. Anti-Fc antibodies detected a high level of EphB2-Fc binding in the EphB2-Fc treated samples (B), while only a low background level of labeling occurred in the Fc-treated controls (A). Colabeling with tubulin β -III (C–E) and glial fibrillary acidic protein (GFAP) (F–H) indicated that EphB2-Fc colocalized with both tubulin β -III-positive axons (E) and GFAP-positive astrocytes (H). Scale bar, 50 μ m.

mutant mice were indistinguishable morphologically from untreated wild type littermates (Figs. 2B–D). LIOH treatment (Fig. 2E) resulted in darkly stained axoplasm, myelin debris, axon swelling, and gliosis in wild type littermates, resembling the optic nerve damage previously observed in a different colony of wild type CD-1 mice after LIOH.³⁴ Axon degeneration induced by LIOH appeared more severe in *EphB2*^{−/−} (Fig. 2F) and *EphB3*^{−/−} (Fig. 2H) mice, as evidenced by more prominent gliosis, more dark myelin debris, swollen fibers, and fewer morphologically normal axons.

Quantitative analysis of axon counts demonstrated that untreated *EphB2*^{−/−} and *EphB3*^{−/−} mice contained numbers of RGC axons comparable with those of untreated wild type littermates (Fig. 3). In wild type littermates, LIOH reduced the axon count to $62 \pm 14\%$ ($P = 0.01$) of the mean value seen in untreated animals (Fig. 3) of the same genotype. *EphB2*^{−/−} and *EphB3*^{−/−} mice exhibited significantly more extensive axonal degeneration compared with wild type littermates. In these mice, the number of surviving morphologically normal axons was reduced to $21 \pm 5\%$ ($P < 0.0001$) and $28 \pm 5\%$ ($P < 0.0001$) of genotype-matched untreated animals, respectively. These data showed a more severe decline in the number of morphologically intact optic nerve axons in animals lacking EphB2 or EphB3 function and suggest that an endogenous

mechanism normally exists to temper axon injury/loss after LIOH. Furthermore, this endogenous process can be modified by EphB protein function, with EphB2 and EphB3 having only a limited ability to fully substitute for each other.

EphB2 Reverse Signaling Partially Rescues Axon Loss after LIOH

In light of potential bidirectional signaling in the Eph/ephrin B system, the mechanism by which *EphB2* participates in moderating optic nerve axon loss induced by LIOH was examined in *EphB2*^{lacZ/lacZ} mice. *EphB2*^{lacZ/lacZ} animals express an EphB2 fusion protein in which the tyrosine kinase and C-terminal domains of EphB2 are replaced by β -galactosidase (β -gal). This EphB2- β -gal fusion receptor is deficient in forward signaling due to the lack of the catalytic tyrosine kinase domain and the postsynaptic density-95/discs large/zona occludens-1 (PDZ)-binding motif. However, it maintains the ability to activate reverse signaling via ephrin-B molecules to which it binds.^{35,43–46}

Measurements of IOP in *EphB2*^{lacZ/lacZ} mice (Fig. 1) before (12.7 ± 0.7 mm Hg) and after LIOH (28.5 ± 1.5 mm Hg) were similar to wild type littermates. The morphology of LIOH-induced axon degeneration in *EphB2*^{lacZ/lacZ} (Fig. 2G) optic

nerve cross-sections appeared more severe than that observed in wild type littermates (Fig. 2E), although not as much as in *EphB2*^{-/-} (Fig. 2F) mice. Quantification of axon loss confirmed that C-terminally truncated *EphB2*^{lacZ/lacZ} mice were significantly less affected after LIOH compared with protein-null mutant *EphB2*^{-/-} animals (Fig. 3). The reduction of intact axon number to $40 \pm 10\%$ ($P < 0.0001$) of untreated controls observed in *EphB2*^{lacZ/lacZ} was intermediate between that of wild type and *EphB2*^{-/-} animals. This finding suggested that in *EphB2*^{lacZ/lacZ} mice, the presence of ephrin-B-mediated reverse signaling (activated by extracellular N-terminus of EphB2) resulted in improved axon survival compared with protein-null *EphB2*^{-/-} animals, in which both EphB2-mediated forward signaling and ephrin-B2-mediated reverse signaling were absent. However, this rescue was incomplete and *EphB2*^{lacZ/lacZ} mice still exhibited more severe axon degeneration than wild type littermates, likely due to the absence of EphB2-mediated forward signaling. Taken together, the data suggested that EphB/ephrin-B signaling in both the forward and reverse directions was involved in the endogenous axon protective process active in LIOH experimental glaucoma.

EphB2 Treatment Slows Glaucomatous Degeneration in Optic Nerve Explants

The in vivo analysis of axon integrity and damage in mutant animals provided evidence that EphB/ephrin-B signaling influences axonal survival after LIOH treatment. As the loss-of-function data implicated both EphB forward signaling and ephrin-B-mediated reverse signaling, we wondered whether manipulation of EphB/ephrin-B signaling via a gain-of-function approach can modulate the severity of axon damage after LIOH.

Explants consisting of optic nerves with retinas attached were cultured and stained with tubulin β -III antibodies as a measure of axon integrity in the ONH region. Untreated control optic nerves from wild type CD-1 mice exhibited robust tubulin β -III staining (Fig. 4A), indicating that this explant culture method adequately supports RGC axon survival for at least 4 days in vitro. In contrast, retina/optic nerve explants cultured 2 days after LIOH treatment (Fig. 4B) and examined after 4 days in vitro displayed evidence of axonal degeneration, with readily apparent axon swelling, aberrant axon trajectories, and large regions of axon drop-out at the ONH. Quantitative analysis showed that the level of tubulin β -III immunoreactivity was significantly reduced by $50 \pm 9\%$ ($P = 0.003$) in LIOH treated retina/optic nerve explants compared with untreated controls (Fig. 4C). Although some degeneration inevitably occurs in any culture system, LIOH treatment demonstrably resulted in more damage than that seen in explants from untreated animals prepared and cultured in the same manner.

Examination of LIOH-treated optic nerve explants revealed that the axon damage and loss observed after 4 days in vitro occurred after placement in culture. Optic nerves harvested 2 days after LIOH and examined immediately with immunohistochemistry exhibited some local axon swellings and defasciculation, but widespread axon loss had not yet become pronounced (data not shown). In contrast, the extent of axon loss was more severe in optic nerves treated with LIOH in parallel but subjected to the additional 4 days of culture. These findings suggest that although IOP elevation is no longer present in vitro after tissue removal from the animal and placement in culture, axonal degeneration in cultured LIOH-treated retina/optic nerve explants continues to progress further.

Using LIOH-treated optic nerve explants, we next investigated the effect of exogenous EphB2 recombinant protein application on axonal survival in vitro. Preclustered EphB2-Fc was added to the culture media, while Fc protein alone was

used as control. Fc-treated optic nerves (Fig. 4D) were morphologically similar to those cultured in media alone (Fig. 4B). In EphB2-Fc-treated optic nerves, axonal swelling and meandering trajectories could still be observed, but a larger number of tubulin β -III-positive axons were preserved (Fig. 4E). Quantification of tubulin β -III staining indicated that the level of immunoreactivity in LIOH-treated optic nerves was significantly improved by $80 \pm 11\%$ ($P < 0.0003$) with EphB2-Fc application compared with Fc alone (Fig. 4F).

To demonstrate EphB2-Fc protein binding within optic nerve explants, we cultured non-LIOH treated nerves for 1 day in the presence of preclustered Fc or EphB2-Fc. Significant binding of recombinant EphB-Fc protein was observed in EphB2-Fc treated nerves (Fig. 5B), compared with the background level in Fc treated controls (Fig. 5A). EphB2-Fc colocalized with both the axonal marker tubulin β -III (Figs. 5C-E) and the astrocytic marker GFAP (Figs. 5F-H), consistent with the expression of ephrin-B in both axons and astrocytes.³¹ These results are also reminiscent of previous studies showing that EphB2-Fc and ephrin-B2-Fc proteins bind to RGC axons after in vivo application in the optic nerve.³⁰

DISCUSSION

Bidirectional EphB/ephrin-B Signaling Promotes Axonal Survival in Mouse Experimental Glaucoma

Several studies have previously demonstrated the upregulation of EphB/ephrin-B expression in glaucoma, including cultured cells from human patients,^{13,29} tissues obtained from a primate glaucoma model,²⁹ DBA/2J mice,³⁰ and LIOH-induced glaucoma in CD-1 mice.³⁴ Eph and ephrin are membrane-anchored proteins and require tight apposition of neighboring cells for activation. Both primate and mouse ONHs are unmyelinated, where RGC axons are directly ensheathed by astrocytes.^{12,47-52} This cytoarchitecture potentially allows for the occurrence of contact-dependent Eph/ephrin signaling to mediate axon-glia and axon-axon communication in response to glaucomatous insults.

Although the upregulation of EphB/ephrin-B signaling in multiple animal models and in cells from human patients suggest that these signaling molecules may represent a constant feature of disease, it was previously unknown whether EphB/ephrin-B signaling is functionally involved and how it may influence pathologic progression. Using the LIOH experimental glaucoma model in several strains of EphB mutant mice, here we present evidence that EphB/ephrin-B signaling is functionally involved in modulating the axonal pathophysiology of glaucoma. In LIOH, laser treatment induces rapid and transient IOP elevation, which returns to baseline by 1 week. Despite IOP normalization, axonal degeneration continues to progress, with approximately 50% of axons remaining at 1 week and only approximately 20% by 1 month.³⁴

Our previous work employing the LIOH model was conducted with CD-1 mice purchased directly from Charles River Laboratories (CRL), where they were maintained as outbred stock. In the present study, the EphB mutant mice of the CD-1 background were maintained for multiple generations initially in Mark Henkemeyer's laboratory at the University of Texas, Southwestern, and then at our facility at the University of California San Francisco. In the earlier work,³⁴ we reported approximately 37,000 axons in the optic nerve of CD-1 mice obtained from the commercial vendor. In the present study, the number of axons in the untreated wild type CD-1 littermates from our colony was found to be higher at approximately 50,000 (Fig. 3). We attribute this difference in optic nerve axon number to a possible colony effect. Despite this

difference, wild type littermates in the present study responded similarly to LIOH as CD-1 mice from CRL. Both exhibited comparable IOP peaks at day one after LIOH and approximately 60% axon survival 1 week after LIOH induction. Genetic protein-null deletions of *EphB2* or *EphB3* both resulted in more severe axonal degeneration, with only approximately 20% and 30% of axons remaining, respectively. These results indicate that EphB2 and EphB3 normally participate in an endogenous process that moderates axon loss after LIOH.

The observation that either EphB2 or EphB3 deficiency can produce a detectable change in axon damage argues that the effect of these EphB proteins is nonredundant. However we did not test for dose dependency by comparing results from EphB2 and EphB3 double-null animals versus single-null animals, due to the difficulty in obtaining sufficient numbers of double mutant animals for study. Although the functional redundancy of various Eph and ephrin family members during development has been widely reported,^{44,53,54} a pathologic situation in adulthood might behave differently. In this context, EphB and ephrin-B expression at the normal adult ONH is low, and only becomes upregulated after induction of experimental glaucoma. The relatively short-term exposure to glaucomatous injury in this experimental model may not allow for significant compensatory action between EphB family members. Furthermore, mice carrying the C-terminally truncated EphB2 exhibited a phenotype intermediate between *EphB2*^{-/-} mice and wild type littermates. This suggests that on one hand, deletion of the EphB2 C-terminal domains renders the animals more susceptible to glaucomatous insults, possibly due to the loss of forward signaling. On the other hand, the remaining ectodomain is able to mediate part of the protective function of EphB2, likely via reverse-signaling through ephrin-Bs. These results are consistent with our previous observation that ephrin-B activation is relatively low in aberrant axons compared with their morphologically normal neighbors.³¹ Another possible explanation for nonredundancy is that EphB2 and EphB3 may act through different mechanisms that converge on axon survival. Some insight could be gained in the future by using cell-specific gene deletion strategies.

EphB2 and *ephrin-B2* have also been found previously to be upregulated in aged DBA/2J mice that exhibit optic nerve degeneration.³⁰ While LIOH is a relatively acute experimental model, the DBA/2J model is spontaneous, slow, and asynchronous. We are currently characterizing a congenic strain of DBA/2J mice deficient in EphB2 to examine whether EphB/ephrin-B signaling plays a role in the progression of glaucoma in DBA/2J animals.

Potential Downstream Mediators of EphB Forward Signaling at the ONH after LIOH

EphB forward signaling principally depends on the catalytic kinase domain, which can act through autophosphorylation as well as phosphorylation of other proteins. A number of effector molecules can be recruited to the receptor to mediate downstream signaling cascades, including Src family kinases and guanine nucleotide exchange factors (GEFs), which in turn activate the Ras/Rho-family GTPases.^{22,55} The C-terminal region can also signal via kinase-independent mechanisms, including protein association with its PDZ-binding motif.

Unpublished data from our laboratory indicate that phosphorylation and presumably activation of Vav2, a GEF for Rho family GTPases, was upregulated at the ONH after induction of LIOH. EphB deficiency appears to lead to diminished Vav2 phosphorylation after LIOH, consistent with the hypothesis that Vav2 may be activated downstream of EphB forward signaling in LIOH-induced glaucoma (data not shown). A re-

cent study⁵⁶ suggests that Vav2/Vav3-deficient mice exhibit an ocular phenotype reminiscent of human glaucoma, and single nucleotide polymorphism analysis identified VAV2 and VAV3 as candidate glaucoma susceptibility loci in Japanese open-angle glaucoma patients. Although the authors proposed that the RGC loss and ONH cupping in these mutant mice are due to the loss of VAV2 and VAV3 function in angle tissue resulting in iridocorneal angle closure and IOP elevation, it is possible that VAV2 and VAV3 may play additional roles in other ocular tissues with relevance to glaucoma, for example at the ONH. Future functional studies are needed to investigate whether Vav may mediate the protective effect of EphB/ephrin-B signaling.

We have also examined components of other signaling pathways that have been implicated in glaucoma, axon degeneration, or interaction with the Eph/ephrin signaling system. Analysis of NFκB⁵⁷⁻⁵⁹ and phosphorylated S6 as a marker of PI3K/mTOR pathway activation⁶⁰⁻⁶² revealed no consistent difference between *EphB2*^{-/-} or *EphB3*^{-/-} and wild type littermates treated with LIOH. The cytokine IL-6⁶³⁻⁶⁶ exhibited a moderate increase at the ONH of both *EphB2*^{-/-} and *EphB3*^{-/-} mice subjected to LIOH (data not shown).

Ephrin-B Reverse Signaling and Alternative Mechanisms

The ephrin-B reverse signaling pathway also involves tyrosine phosphorylation of the C-terminus by Src family kinases, or protein association with the PDZ-binding domain. EphB ectodomain can activate reverse signaling by ephrin-B molecules without the participation of the intracellular portion of EphB, which may account for the partial effect of the truncated EphB2 protein we observed in *EphB2*^{lacZ/lacZ} mice. Alternatively, EphB ectodomain may also interact with other molecules in *cis*.^{21,22} For example, the facilitation of N-methyl-D-aspartate (NMDA) receptor clustering at synapses is kinase-independent and mediated by the extracellular N-terminus of EphB2.⁶⁷ In addition, extensive interplay between Eph/ephrin family members and components of other signaling pathways has been described.⁵⁴ The possibility remains that the observed function of EphB proteins could be mediated by either *cis* or *trans* interaction with other pathways.

EphB/ephrin-B Signaling as a Potential Therapeutic Target

Using a gain-of-function approach, we demonstrated that exogenous application of EphB2 recombinant protein attenuated glaucomatous axonal degeneration in vitro. These findings potentially open up a new avenue of research in glaucoma intervention. Studies using phage display strategies have identified high-affinity peptides that specifically bind individual EphA or EphB receptors.⁶⁸⁻⁷⁰ Although such peptides have so far been used as selective antagonists to EphB signaling, the multimeric forms of these compounds could potentially induce receptor clustering and promote forward signaling. With the development of novel selective reagents, it may be possible to investigate the role of individual EphB and ephrin-B molecules and explore their therapeutic potential in vivo.

Acknowledgments

The authors thank Ivy Hsieh (Cell Imaging Laboratory of Veterans Affairs Medical Center, San Francisco) for PPD staining, and Tony Tran (UCSF) for mouse colony management.

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