Upregulation of EphB2 and ephrin-B2 at the Optic Nerve Head of DBA/2J Glaucomatous Mice Coincides with Axon Loss

Juan Du,1 Tony Tran,1 Christine Fu,1,2 and David W. Sretavan1,2,3

METHODS. In situ hybridization and cell-type–specific immunolabeling were performed on ONH sections from DBA/2J mice (3 to 11 months old) and C57Bl/6Ncr mice (10 months old). EphB2-Fc and ephrin-B2-Fc chimeric proteins were applied to adult RGC axons in vitro and in vivo at the ONH to demonstrate protein binding on axons. EphB2-Fc or control Fc protein was applied in a bath or locally to axons preloaded with the calcium indicator Fluo-4-AM, and changes in intra-axonal calcium were determined.

RESULTS. EphB2 and ephrin-B2 were specifically upregulated at the ONH of DBA/2J mice starting at 9 months of age, but not in age-matched C57Bl/6Ncr mice or in DBA/2J animals that did not have axon loss. EphA4 was also present at the ONH, but no difference in expression was detected between unaffected and affected animals. EphB2 was expressed by F4/80+, MOMA2+, ED1+ macrophage-like cells, ephrin-B2 was expressed by Iba-1+ microglia and GFAP+ astrocytes, whereas EphA4 was expressed by GFAP+ astrocytes. EphB2-Fc and ephrin-B2-Fc protein bound to RGC axons in culture and to ONH RGC axons in vivo. Adult RGC axons in vitro elevated intra-axonal calcium in response to EphB2-Fc but not to control Fc protein.

CONCLUSIONS. The expression of Ephb2 and ephrin-B2 is upregulated at the ONH of glaucomatous DBA/2J mice coinciding with RGC axon loss. The direct binding of EphB2 and ephrin-B2 on adult RGC axons at the ONH and the ability of EphB2 to elevate intra-axonal calcium indicate that these proteins may affect RGC axon physiology in the setting of glaucoma and thus affect the development or progression of the disease. (Invest Ophthalmol Vis Sci. 2007;48:5567–5581) DOI:10.1167/iovs.07-00442

The molecular mechanisms leading to retinal ganglion cell (RGC) axon damage and the progressive loss of RGCs in glaucoma are of substantial interest. Both clinical observations and experimental evidence indicate that the initial pathogenic insults are likely to affect RGC axons and occur within the lamina cribrosa of the optic nerve head (ONH).1–5 Mechanical or ischemic mechanisms of damage have been proposed in which pressure transmitted to the lamina cribrosa results in axon compression or affects the local vasculature, leading to ischemic injury.6–8 Based on work in vitro, it has also been suggested that specific ONH cell populations directly contribute to axon damage. One proposed mechanism involves ONH astrocytes that have been shown in culture to express inducible nitric oxide synthase (NOS)-2 in response to tumor necrosis factor (TNF)-α and other cytokines.9,10 The exact role of NOS-2 awaits further study, as this molecule has not been found in the ONHs of animals with glaucoma induced by episcleral vein hypertonic saline injection.11 Nevertheless, cellular and extracellular matrix reorganization has been clearly shown in the diseased ONH,12,13 and the notion that intrinsic cell populations may express factors or molecules that contribute to axonal damage is worth consideration. Given the proposed importance of the ONH in glaucoma, candidate molecules that potentially directly affect the development or progression of axon loss in this disease should fulfill several basic criteria. First, their presence should be demonstrated locally within the ONH region. Second, their appearance should coincide roughly with the onset of axon loss. Third, RGC axons must be able to respond to the direct application of the molecules of interest and demonstrate an alteration in basic physiology.

Recent studies indicate that fundamental aspects of RGC axon development and their behavior after optic nerve injury are governed by the Eph and ephrin families of receptor tyrosine kinases and ligands.14–16 Not only do embryonic RGC axon growth cones read signals provided by Eph and ephrin proteins as they navigate to reach the appropriate central nervous system (CNS) targets,17,23 but RGC axons in the adult visual system also maintain an ability to respond to these protein families, after their upregulation in the setting of traumatic injury.24,25 After optic nerve damage, RGC axons respond with a robust sprouting response that is mediated by the EphB3 displayed on the surface of macrophages recruited to the site of nerve damage. The elimination of EphB3 gene function in mice significantly reduces this RGC axon plasticity. Of note, other members of these protein families such as ephrin-B2 and EphA4 have also been shown to govern the ability of damaged corticospinal axons to regrow after spinal cord injury,26–28 suggesting that Eph and ephrin proteins are intimately involved in axonal response to traumatic injury throughout the nervous system.

In this study, we investigated the possibility that Eph and ephrin proteins also play a role in more chronic progressive forms of axonal injury such as glaucomatous optic neuropathy. The results demonstrated that the ONH region in glaucomatous DBA/2J mice was characterized by the highly localized, upregu-
lected expressions of EphB2 in macrophage-like cells and of ephrin-B2 in microglia and astrocytes. EphB2 and ephrin-B2 expression correlated closely with the presence of disease, and their upregulation roughly coincided with the reported onset of axon loss. In vivo, RGC axons within the ONH bound EphB2 and ephrin-B2, and in vitro, the local application of recombinant EphB2 protein onto individual RGC axons triggered elevations of intra-axonal calcium. Together, these data demonstrate that the glaucomatous mouse ONH contains multiple cell populations that can interact with one another and with RGC axons via Eph and ephrin signaling. Moreover, the appearance of this potential signaling network correlated specifically with glaucoma, and EphB2 can lead to fundamental alteration in calcium management in adult RGC axons.

**Methods**

**Animals**

DBA/2J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or obtained from Philip Horner (University of Washington, Seattle, WA) and housed in animal facilities at University of California San Francisco (UCSF). C57Bl/6Ncrl mice were obtained from Charles River Laboratories (Wilmington, MA). All research was conducted under protocols approved by the UCSF Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Optic Nerve Tissue Processing**

DBA/2J female mice at 3, 8, 9, and 10 to 11 months of age and C57Bl/6Ncrl female mice at 10 months of age were anesthetized with an overdose of pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the eyes were enucleated along with the optic nerve. The anterior segment together with the lens and the vitreous were then removed, and the remaining eye cup and optic nerve were further postfixed in 4% paraformaldehyde at 4°C overnight, followed by cryoprotection with 17% sucrose. Tissue blocks were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and were used for immunostaining or in situ hybridization.

**Verification of RGC Axon Loss**

Although most DBA/2J animals exhibit glaucomatous damage and axon loss beginning at 9 to 10 months of age, it is known that up to a third of animals, even at 12 months of age may not have evidence of disease.58 RGC axon loss in individual animals from both the Jackson Laboratory and the University of Washington was determined by the presence or absence of optic disc cupping, as visualized in histologic sections through the ONH. In addition, after obtaining the ONH region that contained the cells that show increased mRNA as a proportion of the total mRNA in each ONH sample was potentially affected by the actual size of the dissected tissue. To account for such variation, a dilution factor of the dissected tissue. To account for such variation, a dilution factor of the dissected tissue. To account for such variation, a dilution factor was calculated for normalization of the total RNA of each sample and to correct the Ct using the formula Ct (corrected) = Ct (read) – log(DF)/log 2. After real-time-PCR analysis, the ONH samples were divided into two groups based on the presence or absence of axon damage in PPD-stained sections of the individual optic nerves. The relative expression of EphB2 and ephrin-B2 in these two groups of samples were compared by using the 2-ΔΔCt method.55

**Immunostaining**

Ten- to 12-μm-thick cryostat tissue sections were air-dried for 20 to 30 minutes and washed with PBS (0.1 M, pH 7.4) twice for 10 minutes each. Sections were then blocked in PBS containing 0.2% Triton X-100 and 10% normal goat serum for 1 hour at room temperature. Primary antibody incubation was performed in PBS, 0.2% Triton X-100, and 2% normal goat serum overnight at 4°C. After 5-minute PBS washes repeated three times, Cy3-conjugated secondary antibody was applied at 1:200 dilution for 2 hours at room temperature. After three 5-minute
PBS washes, slides were mounted in medium (Vectashield; Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI). Fluorescence imaging analysis and capture were performed on an inverted confocal laser microscope (LSM 5 Pascal; Carl Zeiss Meditec, Inc., Thornwood, NY).

The following antibodies were used: rat anti-mouse CD11b (1:15; Serotec, Indianapolis, IN); rat anti-mouse CD 31 (PECAM-1; 1:100; BD Biosciences, San Jose, CA); rat anti-mouse CD4 (1:20; Chemicon, Temecula, CA); hamster anti-mouse CD69 (1:50; Chemicon); mouse anti-rat monocytes/macrophage (ED1; 1:50; Chemicon); rat anti-mouse macrophages/microglia (MOMA-2; 1:15; Chemicon); mouse anti-oligodendrocytes (RIP; 1:50; Chemicon). Antibody labeling was visualized using secondary antibodies conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). The binding of recombinant Fc fusion proteins in vitro or in vivo was detected using Cy3 conjugated anti-human IgG Fcγ (1:200, Jackson ImmunoResearch).

PPD Staining

PPD staining was performed as previously described, to visualize degenerating RGC axons in the optic nerve.25 Briefly, segments of the optic nerves beginning approximately 2 mm behind the globe were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde at 4°C. The tissue was further postfixed with 1% OsO4 in PBS for 2 hours at room temperature, followed by 10-minute washes with 0.1 M sodium acetate buffer for repeated three times. The sections then were stained with 2% uranyl acetate in sodium acetate buffer for 1 hour at room temperature. After buffer washes, the sections were dehydrated in a graded ethanol series (40%-100%), and infiltrated with propylene oxide-812 resin (1005 Embed 812; EMS, Fort Washington, PA). The sections were embedded with fresh 100% 812 resin in molds and polymerized in a 60°C oven for 36 hours. One-micron-thick sections were collected and stained with 1% PPD in 1 part methanol/1 part isopropanol for 30 minutes, washed in fresh isopropanol twice, and coverslipped.

Retinal Explant Culture

Adult retinal explants were cultured in vitro, as previously described.25,34 Ten- to 11-month-old DBA/2J mice or 6- to 12-month-old C57Bl/6NCrl mice were anesthetized as described earlier and kept warm during the procedure with heating pads. Injections of recombinant proteins into the ONH were performed in a manner similar to protein injection into the optic nerve, which is described elsewhere.25 Briefly, using a surgical microscope, the conjunctiva was opened at the lower temporal quadrant, blood vessels were cauterized, and the intraocular muscles were retracted. The conjunctiva was then further dissected, and the ONH region was exposed by carefully pushing away sinus tissues with a pair of blunt forceps. The optic nerve sheath surrounding the ONH was dissected with a microfabricated optic nerve sheath microknife to assist in the visualization of the intended site of protein injection. A glass micropipette loaded with recombinant EphB2-Fc, ephrin-B2-Fc, or Fc protein (all at 2–4 μg/μl) was inserted into the ONH region within 200 μm immediately behind the globe. Protein was injected into the ONH using pressure pulses (14 psi and 20 ms duration) delivered by a picospritzer (Parker Hannifin Corp., Fairfield, NJ). After the injection, 1% lidocaine and neomycin ointment were applied to the experimental eyes. Eyes with intraoperative bleeding or postoperative infection were excluded from analysis. The results from at least three eyes were analyzed for each protein injected. Mice were perfused with 4% paraformaldehyde 4 to 5 days after protein injection. The optic nerves were removed and 16-μm-thick cryostat tissue sections were obtained. EphB2-Fc, ephrin-B2-Fc, and Fc protein binding was visualized with the protocol described above described earlier.

Calcium Imaging

Axons from adult retinal explant cultures were loaded with 5 μM fluo-4-AM (Invitrogen, Carlsbad, CA) at 37°C, 95% O2, 5% CO2, for 1 hour. Explants were then washed two to three times and further incubated in fresh growth medium (without fluo-4-AM) for another 20 minutes. Cultures were then placed on a heated microscope stage maintained at 37°C in a CO2 environment. For experiments involving bath application, recombinant proteins were first mixed with BSA (1%) and anti-human IgG Fcγ antibody (1:50) in Neurobasal-A medium (Invitrogen). EphB2-Fc protein was applied to achieve final concentrations in a bath of 1.0, 1.4, 3.6, 7.2, or 14.0 μM. Fc protein was applied to achieve a final concentration in a bath of 14.0 μM. At each concentration, the calcium responses from 5 to 19 axons were studied.

Studies involving the local delivery of EphB2-Fc or Fc recombinant protein onto axons were performed as previously described,24,55,56 with minor modifications. Briefly, the culture media were covered with prewarmed mineral oil (Fisher Scientific) to prevent evaporation. Recombinant protein was prepared as before with the addition of 1:50 dextran-conjugated Texas red (Invitrogen) as a fluorescence indicator. Five microliters of EphB2-Fc (7.2 μM) or Fc (14 μM) was loaded into glass micropipettes. The tip of the glass micropipettes were placed ~30 μm away from the axons under study, and angled almost parallel to the axon shaft, to minimize disturbing the axons during protein delivery (see Fig. 7A). Protein was delivered locally onto axons using repeated pressure pulses of 4 to 9 ms duration at 2-Hz intervals and at 9 psi via a picospritzer. Observation of the red fluorescent indicator mixed in with the test protein verified protein delivery to the axon. The axon segments chosen for study were located 1 mm or greater
away from the explanted retinal tissue. Fluorescence images were captured at 5- to 15-second intervals at 488 nm excitation and 525 nm emission using a CCD camera (PXL2, Photometric or RETIGA EXi, Qimaging, Surrey, BC, Canada; with Deltavision image acquisition software; Applied Precision, Issaquah; or Simple PCI Compix Inc., Sewickley, PA). Baseline fluorescence images of axons were collected for 2 to 3 minutes, followed by protein application during which fluo-4-AM fluorescence was collected for another 10 to 15 minutes. Data images were processed using NIH image J software. The intra-axonal calcium level was measured as the mean gray value of an average of at least three different fixed areas along the axon segment studied. The change of fluo-4-AM fluorescence after application of EphB2 or Fc was normalized as F/F0 and presented as a percentage, in which F0 was the baseline fluo-4-AM fluorescence and F was the change of fluo-4-AM fluorescence with time. Statistical analysis was performed using ANOVA followed by Student’s t-test, and a P < 0.01 was considered significant.

**RESULTS**

**Localized Upregulation of EphB2 and ephrin-B2 at the ONH**

Candidate genes that encode molecules known to regulate the in vivo behavior of developing RGC axons, govern adult RGC axon plasticity after optic nerve injury, or modulate axonal regeneration after spinal cord injury were screened for specific expression at the ONH of glaucomatous DBA/2J mice (Table 1). Results from this analysis showed that while changes in expression for the majority of genes screened were not detected, there was specific localized upregulation of EphB2 (Fig. 1A) and ephrin-B2 (Fig. 1C) at the ONH region of DBA/2J mice at 9 to 11 months of age. The upregulation of both genes was highly specific for the immediate ONH region (arrow) and was not evident in more central portions of the optic nerve (asterisk). No signal was detected when the sense control probe for EphB2 (Fig. 1B) or ephrin-B2 (Fig. 1D) was used.

**Correlation of EphB2 and ephrin-B2 Upregulation with RGC Axon Loss**

In DBA/2J mice, the expression of disease in the form of RGC axon loss is known not to be fully penetrant, and about one half of the animals at 9 to 10 months of age and one third of the animals at 12 months of age do not develop disease. EphB2 and ephrin-B2 upregulation at the ONH was detected in the eyes of 10- and 11-month-old DBA/2J animals that exhibited

---

**Table 1. Expression of Axon Guidance Molecules and Receptors**

<table>
<thead>
<tr>
<th>Molecules (mRNA)</th>
<th>10-Month-Old DBA/2J</th>
<th>3-Month-Old DBA/2J</th>
<th>10-Month-Old C57Bl/6NCrl</th>
<th>3–6-Month Old C57Bl/6NCrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCL</td>
<td>ONH</td>
<td>ONH</td>
<td>ONH</td>
</tr>
<tr>
<td>EphA4</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ephrin-B1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ephrin-B2</td>
<td>+</td>
<td>+++†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ephrin-B3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EphB2</td>
<td>+</td>
<td>+++†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EphB3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EphB4</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slt1</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Slt2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slt3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Robo1</td>
<td>+</td>
<td>+/-</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Robo2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sema5A</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Netrin-1</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

A blank space indicates that analysis was not performed. Expression levels: high, +++; moderate, +++; low, +; faint, +/−; not detected, −. GCL: ganglion cell layer; ONH: optic nerve head

* See Reference 32.
† Expressed in different cell types.
EphB2, ephrin-B2 Upregulation and Axon Loss in Glaucoma

FIGURE 2. Spatial and temporal patterns of EphB2 and ephrin-B2 mRNA upregulation at the ONH. (A) Low-magnification view showing upregulated EphB2 mRNA expression at the ONH of an eye that showed clear optic disc cupping (arrow) in a 10-month-old DBA/2J mouse. Visualized by alkaline phosphatase histochemistry. (B) EphB2 mRNA upregulation was not detected at the ONH in an eye that showed no evidence of optic disc cupping in an 11-month-old DBA/2J mouse. (C) Low-magnification view showing upregulated ephrin-B2 mRNA expression at the ONH of an eye that showed clear optic disc cupping (arrow) in an 11-month-old DBA/2J mouse. (D) ephrin-B2 mRNA upregulation was not detected at the ONH in an eye that showed no evidence of optic disc cupping in an 11-month-old DBA/2J mouse. (E) PPD-stained tissue cross-section of the optic nerve from the eye in (A). Evidence of axon damage (dark profiles) can be seen. (F) PPD-stained tissue cross-section of the optic nerve from the eye in (B). No evidence of axon damage is observed. (G) PPD-stained tissue cross-section of the optic nerve from the eye in (C). Evidence of axon damage can be seen. (H) PPD-stained tissue cross-section of the optic nerve from the eye in (D). No evidence of axon damage is observed. (All sections showing PPD staining, E–H, were obtained from the mid-central regions of the optic nerve.) (I) High-magnification view of EphB2 mRNA expression at the ONH of the same eye as in (A) (10 months DBA/2J). The two arrows indicate the width of the ONH region. (J) High-magnification view of EphB2 mRNA expression at the ONH of the same unaffected eye shown in (B) (11 months DBA/2J). Arrows: examples of cells with faint EphB2 hybridization signal. (K) High-magnification view of ephrin-B2 mRNA expression at the ONH of the same eye as in (G) (11-month-old DBA/2J). Arrows: width of the ONH region. (L) High-magnification view of ephrin-B2 mRNA expression in the same unaffected eye as in (D) (11-month-old DBA/2J). Arrows: examples of cells with faint ephrin-B2 hybridization signal. (M) High-magnification view of EphB2 mRNA in situ hybridization pattern at the ONH of a 10-month-old C57Bl/6NCrl mouse. (N) EphB2 mRNA in situ hybridization pattern at the ONH of a 3-month-old DBA/2J mouse. (O) EphB2 mRNA in situ hybridization pattern at the ONH of an 8-month-old DBA/2J mouse. (P) In situ hybridization using an EphB2 sense control strand. (Q) High-magnification view of ephrin-B2 mRNA in situ hybridization pattern at the ONH of a 10-month-old C57Bl/6NCrl mouse. (R) ephrin-B2 mRNA in situ hybridization pattern at the ONH of a 3-month-old DBA/2J mouse. (S) ephrin-B2 mRNA in situ hybridization pattern at the ONH of an 8-month-old DBA/2J mouse. (T) In situ hybridization using an ephrin-B2 sense control strand. Scale bars: (A–D) 100 μm, (E–H) 20 μm; (I–T) 50 μm.

evidence of axon loss (Figs. 2A, 2C) as documented by optic disc cupping (arrows) and PPD staining of degenerating optic nerve axons (Figs. 2E, 2G). The same pattern of EphB2 or ephrin-B2 upregulation was not observed in animals that did not exhibit optic disc cupping (Figs. 2B, 2D) or did not have evidence of axon loss by PPD staining (Fig. 2F, 2H). At higher magnifications, EphB2 and ephrin-B2 gene upregulation at the ONH of eyes with optic disc cupping was observed to occur in relatively dense cell populations occupying the entire width of the ONH (Figs. 2I, 2K; arrows). This pattern of EphB2 and ephrin-B2 mRNA upregulation was not detected in the ONH of DBA/2J animals of similar ages that did not have RGC axon loss and optic disc cupping (Figs. 2J, 2L). Likewise, EphB2 and ephrin-B2 mRNA upregulation was not detected in 10-month-old C57Bl/6NCrl animals without glaucomatous changes (Figs. 2M, 2Q). In hybridization studies using alkaline phosphatase histochemistry for visualization, we noted that the ONH regions in unaffected DBA/2J and in 10-month-old C57Bl/6NCrl mice contained a sparse and relatively faint hybridization signal (Figs. 2J, 2L, 2M, 2Q, arrows). However, this pattern was quite
Table 2. Correlation of Upregulated Patterns of EphB2 and ephrin-B2 Expression with Optic Disc Cupping

<table>
<thead>
<tr>
<th>Optic Disc Cupping*</th>
<th>Eyes (n)</th>
<th>Sections with mRNA Upregulation (n)</th>
<th>Sections without mRNA Upregulation (n)</th>
<th>% Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphB2 Yes</td>
<td>39</td>
<td>107</td>
<td>105</td>
<td>Sensitivity 100%</td>
</tr>
<tr>
<td>No</td>
<td>18</td>
<td>48</td>
<td>0</td>
<td>Specificity 100%</td>
</tr>
<tr>
<td>ephrin-B2 Yes</td>
<td>9</td>
<td>27</td>
<td>27</td>
<td>Sensitivity 100%</td>
</tr>
<tr>
<td>No</td>
<td>15</td>
<td>35</td>
<td>35</td>
<td>Specificity 100%</td>
</tr>
</tbody>
</table>

* Only eyes with evidence of significant optic disc cupping were included in this analysis.

distinct from the highly specific EphB2 and ephrin-B2 upregulation that occurred throughout the ONH of glaucomatous DBA/2J mice.

EphB2 and ephrin-B2 upregulation at the ONH was observed in DBA/2J mice beginning at 9 to 10 months of age, coinciding with the reported onset of RGC axon loss in this mouse strain.29,37 EphB2 and ephrin-B2 mRNA upregulation at the ONH was not detected in younger DBA/2J animals at 3 (Figs. 2N, 2R) and 8 (Figs. 2O, 2S) months of age. EphB2 and ephrin-B2 upregulation at the ONH was also present in 11-month-old affected DBA/2J mice (Figs. 2I, 2K, 1A, 1C), indicating that these members of the EphB and ephrin-B protein families were likely present at the ONH for several months after the onset of RGC axon loss. Together, the spatial and temporal characteristics of EphB2 and ephrin-B2 upregulation demonstrated that the increased expression of these molecules occurred specifically in the key ONH region for glaucomatous disease during relatively early stages of RGC axon damage.

**EphB2 and ephrinB2 mRNA Upregulation as Indicators of RGC Axon Loss**

A review of the entire set of EphB2 and ephrin-B2 mRNA in situ hybridization experimental data was conducted to determine the accuracy of EphB2 or ephrin-B2 mRNA upregulation as indicators of glaucomatous RGC axon loss in DBA/2J mice (Table 2). This analysis excluded cases in which the technical quality of the in situ hybridization was deemed not to be adequate based on internal controls.

For EphB2, 39 eyes of DBA/2J mice 9 to 11 months of age had evidence of RGC axon loss manifesting as easily identifiable optic disc cupping. From these 39 eyes, 107 tissue sections were processed for EphB2 in situ hybridization. An upregulated pattern of EphB2 mRNA expression at the ONH was detected in 105 tissue sections, indicating that the upregulation of EphB2 at the ONH is a good molecular marker of glaucomatous damage, having a sensitivity of 98.1% in disease detection. In addition, 18 eyes of animals 9 to 11 months of age that had no evidence of RGC axon loss or optic disc cupping were also processed to produce 48 tissue sections for EphB2 mRNA detection. None of these tissue sections showed obvious upregulation of EphB2 mRNA, giving a detection specificity of 100%.

For ephrin-B2, nine eyes of DBA/2J mice 9 to 11 months of age had evidence of RGC axon loss and clear optic disc cupping. In the 27 tissue sections from these eyes processed for ephrin-B2 in situ hybridization, an obvious pattern of ephrin-B2 mRNA upregulation at the ONH was present in all 27 sections, giving a sensitivity of 100% for detection of glaucomatous damage. Thirteen eyes of DBA/2J mice 9 to 11 months of age that had no evidence of RGC axon loss or optic disc cupping were processed to produce 35 tissue sections for ephrin-B2 mRNA detection. None of these 35 tissue sections showed obvious upregulation of ephrin-B2 mRNA, providing a detection specificity of 100%. This analysis revealed that the appearance of upregulated patterns of EphB2 and ephrin-B2 mRNA expression at the ONH was correlated to a very high degree with the presence of glaucomatous RGC axon damage.

**Quantitative Assessment of mRNA Upregulation**

A quantitative assessment of EphB2 and ephrin-B2 mRNA upregulation at the ONH of glaucomatous animals was performed by using real-time PCR. The region of the ONH including the site of the upregulated patterns of EphB2 and ephrin-B2 expression were harvested from 10- to 11-month-old DBA/2J mice and subjected to real-time PCR analysis. The distal portions of the optic nerves from the same samples were also collected, immersion fixed, and processed for PPD staining for assessment of axon damage. Real-time-PCR
optic nerve crush (ONC) injury in a 10-month-old DBA/2J animal. (mice. proven to have axon damage (mRNA analysis conducted on ONH samples that were subsequently revealed that EphB2 mRNA in these samples were on average eightfold more abundant (range, 4.6–13.8-fold) than in samples with no evidence of axon damage (n = 10; Fig. 3A). The ONH samples used for EphB2 mRNA analysis were also used for real-time–PCR analysis of ephrin-B2 mRNA. The results revealed that ephrin-B2 mRNA in the samples with axon damage were on average 5.2-fold more abundant (range, 3–9-fold) than in cases with no evidence of axon damage.

FIGURE 4. Cellular colabeling using EphB2 in situ hybridization and cell-specific antibody immunostaining. (A) Low-magnification view of the ONH region from an affected 11-month-old DBA/2J mouse labeled with the macrophage marker anti-MOMA2 (red) and EphB2 FISH (green). (B) Higher-magnification view of EphB2 hybridization signal in a group of cells from the boxed region in (A). (C) Anti-MOMA2 immunoreactivity in the group of cells shown in (B). (D) Merged image of (B) and (C) demonstrating the coexpression of EphB2 mRNA and MOMA2 in the same cells. (E) Low-magnification view of the ONH region from an affected 10-month-old DBA/2J mouse labeled using the macrophage marker anti-F4/80 (red) and EphB2 FISH (green). (F) Higher-magnification view of EphB2 hybridization signal in a group of cells from the boxed region in (E). (G) Anti-F4/80 immunoreactivity in the group of cells shown in (F). (H) Merged image of (F) and (G) demonstrating the coexpression of EphB2 mRNA and F4/80. (I) Low-magnification view of the ONH region from an affected 10-month-old DBA/2J mouse labeled using the astrocyte marker GFAP (red) and EphB2 FISH (green). (J) Higher-magnification view of EphB2 hybridization signal in a group of cells from the boxed region in (I). (K) Anti-GFAP immunoreactivity in the group of cells shown in (J). (L) Merged image of (J) and (K) demonstrating the expression of EphB2 mRNA and GFAP in different cell populations. (M) Low-magnification view of the ONH region from an affected 10-month-old DBA/2J mouse labeled using the microglia marker Iba-1 (red) and EphB2 FISH (green). (N) Higher-magnification view of EphB2 hybridization signal in a group of cells from the boxed region in (M). (O) Iba-1 immunoreactivity in the group of cells shown in (N). (P) Merged image of (N) and (O) demonstrating the expression of EphB2 mRNA and Iba-1 in different cell populations. (Q) The oligodendrocyte marker RIP1 was absent from the ONH (✱) of affected DBA/2J mice. Anti-RIP1 immunoreactivity was present in more central parts of the optic nerve. (R) Anti-PECAM immunoreactivity was present in the endothelial cells at the ONH and was distinct from the pattern of EphB2 expression. (S) CD4+ T cells were not observed at the ONH of affected DBA/2J mice. (T) Activated CD69 T cells were not observed at the ONH of affected DBA/2J mice. (U) Absence of ED1+ macrophages at the ONH of affected 10-month-old DBA/2J mice. (V) Higher-magnification view of the ONH in (U) showing lack of ED1 immunoreactivity. (W) ED1+ macrophages recruited to the site of an optic nerve crush (ONC) injury in a 10-month-old DBA/2J animal. (X) Higher-magnification view of ED1+ macrophages in (W). Scale bars: (A, E, I, M) 100 μm; (B–D, F–H, J–L, N–P) 20 μm; (Q–T, V, X) 50 μm; (U, W) 200 μm.
Thus both qualitative data from in situ hybridization studies and the quantitative assessment of EphB2 and ephrin-B2 mRNA abundance by real-time PCR supported the upregulated presence of mRNA encoding these genes at the ONH correlated with the presence of optic nerve axon damage.

**EphB2 Expression by Cells with Macrophage-like Properties**

Eph and ephrin proteins play fundamental roles in many cell biological events, in part due to their unusual property of bidirectional signaling.\(^{38-40}\) Binding between an Eph and ephrin molecule leads to the activation of signaling cascades in both the cells expressing Eph and those expressing ephrin. Thus, these molecules are not adequately described solely as either a receptor or ligand and in fact can function simultaneously as both. A substantial body of literature has suggested that the presence of an Eph on a cell and an ephrin on an opposing cell surface is a strong indication of Eph-ephrin-mediated signaling between the two cell types.\(^{14-16}\) Thus, as a first step, we identified the cellular origin of Eph and ephrin gene upregulation at the ONH of glaucomatous animals.

Fluorescence in situ hybridization (FISH) was combined with immunostaining using cell-type-specific markers in DBA/2J ONH tissue sections to determine the cellular origin for EphB2 expression. The results showed that the cells expressing Ephb2 mRNA also expressed the markers for mature macrophages MOMA-2 (Figs. 4A–D) and F4/80 (Figs. 4E–H). A small minority of cells expressing Ephb2 also expressed CD11b (data not shown), a cell surface integrin protein expressed by cells of the monocytic lineage.\(^{41,42}\) Ephb2 cells did not express the astrocytic marker GFAP (Figs. 4I–L) nor did they express Iba-1 (Figs. 4M–P), a common marker for microglia.\(^{43,44}\) Experiments using the oligodendrocyte marker RIP-1 showed no anti-RIP-1 immunostaining in the ONH of affected DBA/2J mice (Fig. 4Q) and the use of anti-PECAM to label endothelial cells showed that the upregulated pattern of Ephb2 expression did not correspond to the endothelial cell network at the ONH (Fig. 4R). Immunostaining using anti-CD4 (Fig. 4S) and anti-CD69 (Fig. 4T) failed to detect the presence of any activated CD4\(^+\) T cells at the ONH or in the optic nerve of glaucomatous DBA/2J animals. (Immunostaining using the same antibodies revealed the expected pattern of staining in the thymus of 21-day-old postnatal mice, data not shown.) Although Ephb2 expressing cells clearly expressed the macrophage markers MOMA2 and F4/80, they did not exhibit another marker of activated macrophages, ED1 (Figs. 4U, 4V). In this respect, the macrophage-like cells at the glaucomatous ONH differed from macrophages encountered after optic nerve trauma that expressed ED1 (Fig. 4W, 4X).\(^{24}\) Together, these results indicate that the cell of origin expressing Ephb2 at the ONH of glaucomatous DBA/2J animals was a macrophage-like cell type. However, this macrophage-like population was distinct from the conventional macrophages that were recruited into the optic nerve after experimental optic nerve crush. Based on the use of cell markers alone, it is not possible to distinguish whether this Ephb2\(^+\) population represented circulating macrophages that had invaded the ONH from the vasculature or were activated microglia that had migrated into the ONH from elsewhere in the optic nerve or retina and taken on macrophage-like characteristics. This subset of MOMA2\(^+\), F4/80\(^+\), ED1\(^+\), and Ephb2\(^+\) macrophage-like cells was not found at the ONH of unaffected DBA/2J animals.

**Shared ephrin-B2 Expression in Microglia and Astrocytes**

Similar colabeling studies were performed to identify the cellular origin for ephrin-B2 expression at the DBA/2J ONH. The results showed that cells expressing ephrin-B2 mRNA colabeled with Iba-1 (Figs. 5A–D), identifying ephrin-B2 expressing cells as microglia. However, we noted that from a quantitative point of view, the number of microglia at the ONH of affected DBA/2J animals was small and that a significant number of ephrin-B2 were Iba-1 negative (Fig. 5D, arrow). Colabeling experiments showed that these Iba-1-negative, ephrin-B2-expressing cells were GFAP positive (Figs. 5E–H) and thus were astrocytes. Iba-1 immunostaining from the data present study did not show obvious changes to the numbers or distributions of microglia in DBA/2J animals with or without evidence of disease.

**Maintenance of EphA4 mRNA Expression by ONH Astrocytes**

While members of the ephrin-B family typically interact only with EphB molecules, some crosstalk with molecules of the EphA subfamily is known to occur.\(^{45-47}\) One such EphA molecule is EphA4, which is known to be capable of binding to ephrin-B proteins including ephrin-B2.\(^{45}\) Furthermore, EphA4 has been shown to be present in astrocytes at the ONH of nonglaucomatous C57Bl/6Ncrl adult mice.\(^{31}\) In DBA/2J animals with documented RGC axon loss, EphA4 expression was detected in GFAP-positive astrocytes (Figs. 6A–D). However, the pattern of EphA4 expression was not apparently different from that observed in unaffected non-glaucomatous DBA/2J animals of similar ages (Figs. 6E–H), and was similar to that in 3-month-old animals (Figs. 6I–L). Thus, in addition to Ephb2 and ephrin-B2, EphA4 was present at the ONH of glaucomatous animals. However, unlike Ephb2 and ephrin-B2, with newly upregulated expression at the ONH that is associated with disease, EphA4 mRNA expression remained largely confined to ONH astrocytes and appeared to be unchanged in diseased versus age-matched control animals.

**EphB2 and ephrin-B2 Binding to RGC Axons In Vitro**

Given that RGC axons at the ONH are all unmyelinated and are in close proximity with microglia, macrophages, and astrocytes that expressed Ephb2 or ephrin-B2 in the setting of glaucoma, it is possible that Ephb2 and ephrin-B2 influences the physiology of adult RGC axons and that this signaling may be a component of disease. To exert any effects on RGC axon physiology, EphB2 and ephrin-B2 must first bind to specific receptors on the surface of RGC axons. As an initial test of EphB2 and ephrin-B2 binding to RGC axons, recombinant chimeric molecules consisting of the extracellular domain of EphB2 or ephrin-B2 fused to human IgG Fc were applied to axons arising from explants of adult mouse retinal tissues. The binding of these EphB2 and ephrin-B2 chimeric molecules on RGC axons was then visualized by the application of fluorescently tagged anti-Fc antibodies. Chimeric Fc molecules have been used extensively to detect specific cell binding in previous studies of Eph and ephrin biology and have been shown to correlate with the presence of specific receptor proteins.\(^{55-58}\) Our results showed that RGC axons in vitro expressed proteins capable of binding EphB2-Fc (Figs. 7A, 7B) or ephrin-B2-Fc (Fig. 7C, 7D) on their axonal surface. The binding appeared as punctate deposits and was similar to that reported for EphB protein binding on embryonic RGC axons.\(^{55}\) Application of the Fc portion (Fc region of human IgG) alone as a control failed to show any specific binding (Figs. 7E, 7F), indicating that binding of EphB2-Fc or ephrin-B2-Fc proteins occurred via the extracellular protein domains of these molecules interacting with presumptive receptor proteins on the surface of RGC axons.
Binding of EphB2 and ephrin-B2 Protein by RGCs at the ONH

An additional test of EphB2 and ephrin-B2 binding on adult RGC axons was performed by injecting EphB2-Fc or ephrin-B2-Fc chimeric proteins directly into the ONH region of adult mice, to determine whether RGC axons within this initial segment of the optic nerve in vivo are capable of binding EphB2 or ephrin-B2. The results showed that the injection of EphB2-Fc or ephrin-B2-Fc led to the specific labeling of linear profiles 1 to 2 μm in diameter, most likely corresponding to RGC axons within the ONH (Figs. 7G, 7H, arrows). Similar injection of Fc control protein alone did not result in such linear patterns of labeling. Instead, a diffuse pattern was observed (Fig. 7I) that probably corresponds to Fc protein present in the extracellular environment in unbound forms. Thus, RGC axons, both in vitro and at the ONH in vivo, selectively bound EphB2 and ephrin-B2 proteins present in their environment. These findings indicate that RGC axons at the ONH are potentially capable of interacting with EphB2 or ephrin-B2 displayed on the surfaces of local macrophage, microglia, and astrocytes.
Alteration of Intra-axonal Calcium in Adult RGC Axons In Vitro by EphB2

To determine whether in addition to binding to RGC axons, EphB proteins can elicit a signaling response in RGC axons, we performed axonal calcium imaging studies after the application of recombinant EphB2-Fc molecules. Introduction of EphB2-Fc recombinant protein into the culture medium resulted in calcium elevation within adult mouse RGC axons, as determined by the calcium indicator dye fluo-4-AM (Fig. 8A). Elevations of intra-axonal calcium were not observed after the application of the Fc protein fragment alone (Fig. 8B). A dose-response relationship was readily observed, with the first detectable calcium elevation occurring after the application of 1.4 μM of EphB2 protein, progressing to a maximum of a 2.5-fold elevation of intra-axonal calcium above baseline after application of 14.0 μM of EphB2 (Fig. 8C). The peak rise in intra-axonal calcium was observed within 2 minutes of EphB2-Fc application with the majority of maximum responses occurring in less than 1 minute (Fig. 8D). The presence of varicosities along the shaft of axons from adult mouse retinal explants (Figs. 8A, 7A–F) has been noted and are similar to those found on adult human and nonhuman primate RGC axons in vivo. We found that intra-axonal calcium elevation in response to EphB2 occurred both along the axon shaft and in the varicosities with a similar time course. Data from axon varicosities are reported in Figures 9C and 9D.

Effect of Local Axonal EphB2 Application on Calcium

To demonstrate that calcium elevations occurred via direct effects of EphB2-Fc protein on axons, we conducted experiments in which EphB2-Fc protein was delivered via a glass micropipette positioned with its tip within 30 μM of an axon segment located 1 to 2 mm away from explanted retinal tissues containing RGC cell bodies (Fig. 9A). Fluorescein was included with the test protein ejected from the glass micropipette, to confirm that reagents delivered in this manner were localized to the axon segment of interest. Such focal EphB2-Fc delivery to axon segments also resulted in intra-axonal calcium elevations (Fig. 9B). This local application-induced calcium response displayed a similar time of response onset in calcium levels (Fig. 9C), compared with the axonal calcium response observed after bath application. A slow decline in intra-axonal calcium from its peak was observed over the course of minutes even with continued local application of EphB2-Fc protein. These results indicated that individual RGC axons from adult retinal explants were sensitive to EphB2 proteins and responded by showing a transient increase in intra-axonal calcium.

**DISCUSSION**

The molecular mechanisms that trigger RGC axon damage and eventual loss in glaucoma are thought to be initiated at the ONH region where elevated intraocular pressure may cause alterations in the local cell and molecular environment. In this study of DBA/2J glaucomatous mice, we examined whether members of protein families that have been implicated in axonal response to acute traumatic injury, including in RGC axons, were also present in more chronic progressive forms of injury, such as glaucomatous neuropathy. We found that EphB2 and ephrin-B2 were upregulated in their expression specifically at the ONH beginning at 9 to 10 months of age, roughly coinciding with the reported onset of RGC axon loss in these animals. Furthermore, EphB2 and ephrin-B2 expression were present only in affected DBA/2J mice, as documented by the presence of optic disc cupping and RGC axon loss, but not in DBA/2J animals of the same age that did not have disease. Double-labeling studies using mRNA in situ hybridization and cell-specific antibody markers showed that EphB2 was ex-
pressed by a distinct population of cells with macrophage-like properties that were not normally present at the ONH, whereas ephrin-B2 was expressed by resident microglia and astrocytes. (These results were obtained in animals from both The Jackson Laboratory and from the University of Washington.) Protein-binding studies demonstrated that RGC axons in vitro and at the ONH in vivo were capable of binding EphB2 protein. In addition, adult RGC axons in

**Figure 8.** Application of EphB2-Fc protein led to elevation in intra-axonal calcium in RGC axons. (A) A series of fluo-4AM fluorescence images of a RGC axon after bath application of EphB2-Fc (7.2 μM). Top right of each panel: time interval between images. Right: pseudocolor representation of the response magnitude. (B) A series of fluo-4AM fluorescence images of an RGC axon after bath application of control Fc protein (14.0 μM). Scale bar, 10 μm. (C) Dose–response relationship between EphB2-Fc protein concentration and the magnitude of intra-axonal calcium elevation (ΔF/F0, %). The numbers of axons tested at each protein concentration is provided at the top of each data column. Right: data from the application of control Fc protein. *Maximum responses to EphB2-Fc that were significantly different from those obtained using Fc control protein. Statistic analysis was performed by ANOVA followed by Student’s t-test. At an EphB2 concentration of 3.6 μM, P = 0.0018; at 7.2 μM, P = 0.00002; and at 14 μM, P = 0.0017. (D) Distribution of the time intervals between EphB2-Fc application and maximum change in intra-axonal calcium. Roughly 50% of the maximum calcium responses occurred within 30 seconds of protein application, and another 35% of the maximum responses occurred between 30 to 60 seconds.

**Figure 9.** Intra-axonal calcium elevation after direct axonal application of EphB2-Fc protein. (A) The relationship between the glass micropipette (P) applying EphB2-Fc protein, the region of the axon under study (boxed area), and the location of the retinal explant tissue (Re). The tip of the micropipette was located approximately 50 μm away from the axon (Ax) and oriented almost parallel to the axon shaft. Boxed region: 100-μm length of axon used for analysis. The retinal explants were located between 1 and 2 mm away from the pipette tip and the axon regions under study. (B) A series of fluo-4AM fluorescence images of an RGC axon after local pipette application of EphB2-Fc (7.2 μM). Top right of each panel: time interval between images. Right: pseudocolor representation of the response magnitude. Scale bar, 10 μm. (C) Time course of intra-axonal calcium changes after continued local application of EphB2-Fc protein. Arrow: the start of EphB2-Fc protein application. Fluo-4AM imaging was performed at 10-second intervals.
events in both cells.\(^{36-40}\) The proposed interaction between astrocytes and microglia is based on the fact that EphA4 can bind to and activate ephrin-B molecules such as ephrin-B2.\(^ {45}\) (B) Diagram illustrating the bidirectional signaling (double-headed arrows) that can occur between RGC axons and macrophages, microglia, or astrocytes at the ONH of glaucomatous DBA/2J mice. The proposed interaction between macrophages and RGC axons is based on the fact that adult RGCs express ephrin-B3\(^ {25}\) to which EphB2 (on macrophages) can bind.\(^ {62}\) This interaction is also based on the present finding that application of EphB2 protein leads to elevation in RGC axonal calcium. Interactions between RGC axons, microglia, and astrocytes are proposed based on the current finding that ephrin-B2 protein binds to RGC axons at the ONH. In addition, an interaction between RGC axons and astrocytes may occur via EphA4 binding to ephrin-B molecules such as ephrin-B3 on RGCs.\(^ {45}\) Note, however, that EphA4-expressing astrocytes are normally present at the ONH of nonglaucomatous DBA/2J mice.

**Ephs and ephrins in Optic Nerve Injury and Neuropathy**

Given that both traumatic optic nerve injury and glaucomatous injury can result in RGC death, these conditions have been suggested to exhibit some molecular features in common. This idea has led to the use of acute optic nerve injury as a model for glaucoma.\(^ {35,52}\) Results from the present study showed that both optic nerve trauma and glaucoma indeed triggered the upregulation of EphB proteins in macrophages at the site of injury. Given that EphB and ephrin-B proteins have also been implicated in regulating axon responses in spinal cord injury,\(^ {76-28,55}\) this protein family may play a key role in various types of axon damage throughout the nervous system.

Significant differences exist, however, between EphB expression after optic nerve injury and in glaucoma. After acute crush injury to the mouse optic nerve, ED1 positive macrophages that are immediately recruited to the lesion site express EphB3 on their cell surface.\(^ {24}\) In vitro and in vivo evidence indicates that traumatically injured RGC axons can respond directly to EphB3, and EphB3 appears to regulate the immediate RGC axon sprouting response that characteristically takes place after optic nerve lesions.\(^ {56-58}\) In glaucomatous optic neuropathy, a different type of MOMA2\(^ { \pm }\), F4/80\(^ { \pm }\), and ED1\(^ { \pm }\) macrophage-like cell that expressed EphB2 was present at the ONH. Since MOMA2\(^ { \pm }\) and F4/80\(^ { \pm }\) cells are not normally present at the ONH, these cells may have been recruited to this site from the vasculature or may represent activated microglia that migrate from within the optic nerve to the ONH. The lack of ED1 expression by these macrophage-like cells and their expression of EphB2, and not EphB3, may indicate that they are indeed of different origin or are in a different state of activation\(^ {59,60}\) than the macrophages present after acute optic nerve injury. Last, glaucomatous injury also involved the upregulation of ephrin-B2 expression in additional ONH cell types such as microglia and astrocytes, cell types known to be present at the ONH in disease.\(^ {9,13,61}\)

**EphB/ephrin-B Signaling at the Glaucomatous Mouse ONH**

The increased molecular expression of EphB2 in ONH macrophages and ephrin-B2 in microglia and astrocytes was superimposed onto a basal level of EphA4 expression by astrocytes and together potentially resulted in a network of cell–cell interactions between macrophage-like cells, microglia, and astrocytes within the glaucomatous ONH (Fig. 10). Given that bidirectional signaling can occur between pairs of cells that express EphB and ephrin-B molecules,\(^ {38-40}\) the present data suggest that a fairly complex set of interactions can take place between ONH macrophage-like cells, microglia, and astrocytes. A further intriguing possibility is that some or all of these ONH cell populations may also affect RGC axons passing through the ONH via EphB/ephrin-B signaling. This possibility is consistent with the demonstration that adult RGCs and their axons expressed axonal surface proteins capable of binding EphB and ephrin-B proteins (Fig. 7).\(^ {24}\) Furthermore, it is supported by the finding that RGC axons at the ONH in vivo are capable of binding EphB2 and ephrin-B2 presented in their environment. Last, the elevations in intra-axonal calcium after bath application or local axonal presentation of EphB2 protein provided functional evidence that adult RGC axons are indeed
EphB2, ephrin-B2 Upregulation and Axon Loss in Glaucoma

Possible Roles of EphB/ephrin-B Signaling in Disease

The results herein suggested that it might be worthwhile to investigate further the potential roles of EphB2 and ephrin-B2, and possibly even EphA4, in the initiation or progression of RGC axon loss in glaucoma. The appearance of EphB2 and ephrin-B2 roughly coincided with the reported onset of axon loss at 9 to 10 months of age in DBA/2J animals and was not found in C57Bl/6Ncrl nonglaucomatous mice of the same age, nor in age-matched DBA/2J mice that did not show RGC axon loss. The spatial patterns of EphB2 and ephrin-B2 upregulation were also precisely localized to the ONH, the suspected site of glaucomatous injury to RGC axons. Together, these findings indicate that the in-migration of macrophage-like cells that expressed EphB2 and that the upregulation of ephrin-B2 on ONH microglia and astrocytes were tightly correlated with the development of disease.

The presence of EphB and ephrin-B molecules in glaucomatous disease was reported in a recent immunohistological study showing the increased presence of EphB1 and ephrin-B1 in cultured optic nerve astrocytes of patients.63 The specific EphB and ephrin-B molecules encountered in that study were different from our current results and were reportedly expressed by astrocytes and not in macrophage or microglia subpopulations, as we found in DBA/2J mice. These results may be due to species differences or the fact that the DBA/2J mouse exhibits a form of pigmentary glaucoma, whereas the primate and human data were obtained from a primate laser-induced experimental glaucoma model and from patients with primary open-angle glaucoma. However, it is worth noting that antibodies against individual Eph and ephrin proteins may not have the desired specificity necessary as reliable probes for individual members of these protein families. Instead Eph and ephrin in situ hybridization combined with binding of Eph and ephrin recombinant proteins to cellular surfaces of interest have typically been used as the standard means of detecting the presence of these molecules. Thus, a characterization of Eph and ephrin gene expression in the primate and human ONH using these two methods may be worth considering particularly given the unusual nuclear localization of EphB1 and ephrin-B1 reported in astrocytes by using anti-EphB1 and anti-ephrin-B1 antibodies in the study of human and primate tissues.63

The data at present do not address how EphB2 and ephrin-B2 might be involved in glaucoma, and it is premature to suppose any causal relationship between disease and Ephs/ephrins at the ONH. The demonstration of calcium elevation in RGC axons exposed to EphB recombinant protein is of potential interest given the known roles of altered calcium handling and neuronal injury and death.64–67 Along this line of reasoning, EphB2 and ephrin-B2 on local ONH cell populations may trigger calcium elevations in RGC axons at the ONH and thus play a role in the onset or the rate of progression of RGC axon loss in glaucoma. In terms of a potential involvement in disease onset, it is worth noting that we did not encounter eyes that showed no optic disc cupping and yet clearly already showed obvious EphB2 and ephrin-B2 upregulation (Table 2). However, since it may be difficult to demonstrate subtle upregulation of gene expression definitively in a disease with variable time of onset between animals, the potential roles of EphB2 and ephrin-B2 in disease initiation should not be ruled out at present.

Alternatively, it is also possible that upregulation of EphB2 and ephrin-B2 signaling represents a local tissue response to axon injury and that EphB2 and ephrin-B2 have a role in supporting RGC axon survival or the remodeling of RGC axons exposed to glaucomatous damage. In support of this notion is the finding that EphB2 at the glaucomatous ONH was expressed by a population of non-ED1 macrophage-like cells, which was unlike the more typical ED1+ macrophages recruited into the mouse optic nerve after traumatic injury.24 Whereas ED1+ macrophages are probably involved in tissue phagocytosis at sites of traumatic damage, the ED1+ macrophages observed at the glaucomatous ONH may represent alternatively activated macrophages that are involved in tissue repair.59,60 In addition, EphB3 that is upregulated after traumatic optic nerve crush injury is required for the sprouting response of injured RGC axons in vivo,24 and serves as a precedent for EphB proteins involvement in axonal remodeling after injury. It is currently unknown whether similar sprouting of RGC axons at the ONH occurs in glaucoma. Last, although Eph and ephrins are well known as potent regulators of RGC axon development and their response to acute injury,14–24 they also play important roles in vasculogenesis.58–71 Although Eph and ephrins have not been shown to directly damage endothelial cells, potential effects on the ONH vasculature should be considered in light of hypotheses of ischemic injury and glaucoma.7 More detailed investigations into the mechanisms by which EphB2 and ephrin-B2 affect RGC axon physiology or the physiology of other ONH cells and whether the development or progression of RGC axon damage is modified in animals with targeted alterations in the function of these proteins may further our understanding of this debilitating disease.

Acknowledgments

The authors thank Philip Horner for DBA/2J mice, Carol Mason for mouse EphA4 plasmid, Juliette Johnson and David Copenhagen for assistance with real-time PCR, and Jennifer LaVail for insightful discussions.

References