

Obstructed Axonal Transport of BDNF and Its Receptor TrkB in Experimental Glaucoma

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PURPOSE. In both animal model systems and in human glaucoma, retinal ganglion cells (RGCs) die by apoptosis. To understand how RGC apoptosis is initiated in these systems, the authors studied RGC neurotrophin transport in experimental glaucoma using acute intraocular pressure (IOP) elevations in rats and chronic IOP elevation and unilateral optic nerve transections in monkeys.

METHODS. Eyes were studied in masked fashion by light and electron microscopy and by immunohistochemistry with antibodies directed against the tyrosine kinase receptors (TrkA, B, and C) and against brain-derived neurotrophic factor (BDNF), as well as by autoradiography to identify retrograde axonal transport of ¹²⁵I-BDNF injected into the superior colliculus.

RESULTS. With acute glaucoma in the rat, RGC axons became abnormally dilated, accumulating vesicles presumed to be moving in axonal transport at the optic nerve head. Label for TrkB, but not TrkA, was relatively increased at and behind the optic nerve head with IOP elevation. Abnormal, focal labeling for TrkB and BDNF was identified in axons of monkey optic nerve heads with chronic glaucoma. With acute IOP elevation in rats, radiolabeled BDNF arrived at cells in the RGC layer at less than half the level of control eyes.

CONCLUSIONS. Interruption of BDNF retrograde transport and accumulation of TrkB at the optic nerve head in acute and chronic glaucoma models suggest a role for neurotrophin deprivation in the pathogenesis of RGC death in glaucoma. (*Invest Ophthalmol Vis Sci.* 2000;41:764-774)

Glaucoma is a slowly progressive optic neuropathy that is characterized by a typical excavated appearance of the optic nerve head and loss of retinal ganglion cells. Older age and the level of intraocular pressure (IOP) are consistent risk factors. Disc excavation is associated with compression and stretching of the connective tissues in lamina cribrosa, the structure that supports ganglion cell axons as they exit the through the optic nerve head.¹⁻³ Retinal ganglion cell (RGC) axons passing through the upper and lower lamina cribrosa die more rapidly, probably because of the lower density of connective tissue support in these areas.

RGC apoptosis has been demonstrated in human eyes with glaucoma, in experimental glaucoma, and after optic nerve transection in monkeys and rats.⁴⁻⁸ Furthermore, evidence from both human eyes and experimental models sug-

gests that glaucoma obstructs anterograde and retrograde axonal transport in RGC axons at the nerve head.⁹⁻¹³ RGC axons undergoing transport obstruction are distended at the nerve head by membrane-bound vesicles known to move bidirectionally between the cell body and axon terminal.^{12,13} We hypothesize that transport obstruction could prevent the arrival at the cell body of molecules required for the health of RGCs and thereby result in apoptotic cell death.

Neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4,5 (NT-4/5) are ideal candidates for factors whose transport interruption could lead to RGC death. Neurotrophins interact with specific receptors, including TrkA, TrkB, and TrkC receptors that are primarily activated by NGF, BDNF, both NT-4/5 and NT-3, respectively. Once activated, neurotrophin/receptor complexes are retrogradely transported to the cell body.¹⁴⁻¹⁸ Neurotrophins, particularly BDNF, are known to influence RGC survival in vitro, both during retinal development and after lesioning.¹⁹⁻³² During development, RGCs are produced in excess, and then neurons whose axons fail to contact the correct target cell die, possibly because of lack of appropriate neurotrophin stimulation.³³⁻³⁶ Although studies of neurotrophins in the retina have focused primarily on their developmental role, adult RGCs also synthesize neurotrophins, exhibit TrkB receptors,^{23,26,28} and respond to BDNF.^{20,22,31,32} Continued neurotrophin dependence among adult RGCs was suggested by a study in which cat RGCs died after their target neurons in the dorsal lateral geniculate nucleus were eliminated with kainic acid.³⁷

These findings suggest the hypothesis that RGC death can be initiated by retrograde axonal transport blockade of neurotrophins that normally arrive from central target cells. In this

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theory, the optic nerve head damage in glaucoma could act as a physiological "transection of the axon." To investigate this possibility, we studied ultrastructural alterations and immunohistochemical localization of BDNF, TrkB, TrkA, and TrkC in optic nerve heads of rats with acute IOP elevation. Furthermore, we localized BDNF and the TrkB receptor in monkey eyes with unilateral, chronic experimental glaucoma and with optic nerve axotomy. Finally, we studied the effect of acute IOP elevation on retrograde transport of radiolabeled BDNF in rats to determine directly if there was disruption of the axonal transport of neurotrophins.

MATERIALS AND METHODS

Rat Acute IOP Elevation Model

Thirty-one adult male brown Norway rats (*Rattus norvegicus*) weighing 250 to 275 g underwent unilateral IOP elevation for 4 hours. Rats were anesthetized by intraperitoneal sodium pentobarbital (50 mg/kg; Sigma Chemical Co., St. Louis, MO) followed by baseline IOP measurements using a Tonopen XL (Mentor, Norwell, MA). A cannula of PE-50 tubing connected to a pressure transducer was placed in the femoral artery to monitor mean blood pressure (BP). A blunt 30-gauge needle connected to a variable height reservoir was placed in the anterior chamber to control IOP. The perfusion pressure (PP) of the eye was defined as the mean BP minus IOP. BP was monitored every 15 minutes, and IOP was adjusted by altering reservoir height to maintain a constant PP. Respiration rate and response to stimuli were monitored to aid in the appropriate administration of supplemental sodium pentobarbital. At PP = 0 mm Hg, there was no visible blood flow in the retina by clinical examination, whereas at PP = 25 mm Hg, blood flow in the retina appeared identical with eyes with normal IOP and did not exhibit either retinal arteriolar narrowing or pulsation. In addition, at PP = 25, no retinal opacity developed, whereas in eyes with PP = 0 opacity was visible. After 4 hours of IOP elevation, animals were euthanatized with pentobarbital.

Among the 31 acute IOP model rats, 23 were studied for ultrastructure or immunohistochemistry of Trk receptors and BDNF. Twelve of these rats had unilateral IOP elevation equal to mean BP (PP = 0) and 11 at 25 mm Hg below mean BP (PP = 25). Eyes from half of each perfusion group were fixed overnight by immersion in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for subsequent embedding in epoxy resin and for light and electron microscopy. The eyes of the other 12 animals were fixed for 4 hours in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, with paraffin embedding for immunohistochemistry.

Eight of the 31 rats were first stereotactically injected with 0.75 μ Ci of 125 I-BDNF (specific activity = 162 μ Ci/ μ g; NEN Life Science Products, Boston MA) into the superior colliculus on both sides of the brain. Within 15 minutes of collicular injection, IOP was elevated. Four rats had IOP elevations in one eye to PP = 0 and the other four were maintained at PP = 25 for 4 hours. These eyes were fixed overnight by immersion in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for subsequent embedding in epoxy resin for autoradiography.

Chronic Experimental Glaucoma Model

Six male juvenile cynomolgus monkeys (*Macaca fascicularis*) were treated with an argon laser one or more times to scar the

trabecular meshwork in one eye, increasing IOP and producing experimental optic nerve injury similar to that of human glaucoma.³⁸ IOP was measured weekly throughout the treatment period with the Tonopen XL (Mentor), and clinical examination of the fundus was performed monthly to monitor the progression of the disease. After 4 months to 2 years, animals were euthanatized by pentobarbital overdose, eyes were rapidly enucleated, and optic nerve heads were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 4 hours. Optic nerve cross sections were embedded in epoxy resin to stage the degree of axon loss as mild, moderate, or severe. This was done by comparison of the remaining neural area to their normal fellow eyes, as well as to a large number of additional, control and glaucoma nerves from previously published experiments in which axon counts were used to validate the three-level severity grading⁷ used here. Optic nerve heads were processed into paraffin for immunohistochemical staining.

Optic Nerve Axotomy Model

Two male juvenile cynomolgus monkeys underwent unilateral intraorbital optic nerve axotomy to produce specific RGC death.³⁹ Fibers were severed posterior to the exit of the central retinal artery and vein to avoid retinal ischemia. Patency of the retinal circulation was verified within 1 week of the procedure by fluorescein angiography. Five months later, animals were killed by pentobarbital overdose, and tissues were prepared as described for glaucoma monkeys.

All animal procedures were approved and supervised by the Animal Care Committee of the Johns Hopkins University School of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transmission Electron Microscopy

Posterior globes of rat eyes (retina, choroid, sclera, optic nerve head, and optic nerve) were postfixed in 1% buffered osmium tetroxide, dehydrated in graded ethanol, and embedded in LX112 epoxy resin (Ladd Research Industries, Burlington VT). The area centered on the optic nerve head was thin-sectioned and stained by 5% uranyl acetate and Reynold's lead citrate before examination in the transmission electron microscope (JEOL 100CX; JEOL USA, Peabody, MA).

Autoradiography

Plastic-embedded segments of the rat eyes containing 3 to 4 mm of retina, the optic nerve head, and up to 3 mm of optic nerve were sectioned at 1 μ m and dipped in undiluted Kodak NTB-2 liquid emulsion (Scientific Imaging Systems/Eastman Kodak Company, Rochester, NY). After 2 months' exposure, these were developed, and the number of grain tracks corresponding to radioemissions were counted in masked fashion from six sections per eye. The number of tracks per square micrometer of tissue was counted in six retinal areas, in the optic nerve head, and in three optic nerve areas on each slide. The mean values for retina, nerve head, and optic nerve were calculated. We recognized that there were some grains overlying the outer retina and a very small number overlying the vitreous humor. Counts were divided into those from the inner and outer retina, including the inner plexiform layer with the inner retina and the inner nuclear layer with the outer retina. Grain counts overlying the outer retina and from three areas

overlying the vitreous on each slide were subtracted from the inner retinal values to yield data that most likely represented specifically transported BDNF. To provide additional confirmation that the observed radioactivity over the inner retina represented axonally transported BDNF, we performed injections into the colliculus in four additional animals. These were made into only the colliculus on one side of the brain (unilateral injection). The injection consisted of a mixture of ^{125}I -BDNF (0.63 μCi) and either 100-fold excess of nonradioactive NGF (2 animals) or 100-fold excess of nonradioactive BDNF (2 animals). It would be expected that a large excess of nonradioactive BDNF would decrease axonal transport of the labeled BDNF, whereas NGF in excess would not compete effectively for the TrkB receptor and labeled BDNF transport would be unaffected.

The ^{125}I -BDNF was unaffected by labeling in its biological behavior, demonstrating specific binding to HEK293T cells transfected with TrkB, but not to untransfected cells (David D. Ginty, personal communication).

Immunohistochemistry

Seven-micrometer longitudinal paraffin sections of rat and monkey optic nerve heads were collected onto Superfrost Plus slides (Fisher Scientific; Pittsburgh, PA) before immunolabeling by the streptavidin-biotin peroxidase technique of Luty et al.⁴⁰ The affinity-purified rabbit polyclonal antibodies were directed against BDNF and against the neurotrophin receptors TrkB, TrkA, and TrkC (Santa Cruz Biotechnology, Santa Cruz, CA). For the colabeling of TrkB and GFAP, an affinity-purified goat polyclonal antibody directed against TrkB was used (Santa Cruz Biotechnology). The antibody recognizing human BDNF was raised against a peptide corresponding to amino acids 128 to 147. Both the rabbit and goat polyclonal TrkB antibodies were directed against an amino acid sequence 794 to 808, located within the intracellular portion of the full-length murine gp145^{TrkB} receptor molecule. The TrkA antibody was raised against a peptide that corresponds to amino acids 763 to 777, adjacent to the carboxyl-terminus of the precursor form of the porcine gp140^{TrkA} receptor molecule, and the TrkC antibody was directed against a peptide corresponding to amino acids 798 to 812 of the precursor form of porcine TrkC, gp140^{TrkC}. TrkB, TrkA, and TrkC antibodies were noncrossreactive with one another, as determined by the supplier with immunoprecipitation. Western blot analysis confirmed the presence of bands at the appropriate molecular weight (data not shown). Primary antibodies were applied at 0.5 to 1.0 $\mu\text{g}/\text{ml}$. Negative controls included nonimmune serum of the same species as the primary antibody at the same protein concentration, primary antibody preincubated with fivefold excess control peptide, and incubation buffer alone. Labeled sections were mounted in glycerol jelly and viewed by Nomarski optics (Zeiss Axioskop; Carl Zeiss Inc., Thornwood, NY).

In addition, a series of rat and monkey optic nerve head sections were colabeled with goat anti-TrkB and a rabbit polyclonal antibody directed against bovine GFAP (Dako Corporation, Carpinteria, CA) to ascertain if TrkB label was localized in astrocytes. For this experiment, goat anti-TrkB was followed by donkey anti-goat biotinylated secondary antibody (Santa Cruz Biotechnology) and Cy3-labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. After reblocking in normal serum, rabbit anti-GFAP was applied followed by fluorescein isothiocyanate (FITC)-labeled goat anti-

rabbit secondary antibody (Santa Cruz Biotechnology). Sections were mounted in Vectashield mounting media (Vector Laboratories, Burlingame, CA) and viewed on a confocal microscope (Zeiss LSM 410; Carl Zeiss Inc.). Images of Cy3-TrkB label were collected using a helium-neon laser at 543-nm excitation, with a 570-nm longpass filter, whereas images of FITC-GFAP were collected using a krypton-argon laser at excitation 488 nm, with a band pass of 505 to 580 nm. Cy3-TrkB images were pseudocolored red and FITC-GFAP green before their superimposition.

Because retinas from the monkey nerve heads had been removed for other studies, additional retinal samples from archival control monkey eyes were labeled with goat anti-TrkB as described above. Epifluorescent images of the Cy3-TrkB label were collected with the Zeiss Axioskop using a rhodamine filter (Carl Zeiss Inc.).

Masked Evaluation of BDNF and TrkB Label in Rat and Monkey Experimental Models

A masked evaluation of control and experimental rat eyes based on their TrkB and BDNF labeling was performed. The staining intensity of the nerve fiber layer (NFL), ganglion cell layer (GCL), superficial optic nerve head, optic nerve head, nonmyelinated zone just behind the globe, and myelinated optic nerve were noted. In normal rat eyes, TrkB label was uniformly present from the retina through the optic nerve head into the optic nerve. For each rat eye, the degree of difference from normal after IOP elevation was graded on a four-level scale from 0 (normal) to 3 (most abnormal). This score summarized both general alterations in the distribution of label and focal label accumulation.

In normal rat eyes and in those with acute IOP elevation, BDNF labeling was barely detectable even with the optimal protocol.

In monkey tissues, a masked examination for BDNF and TrkB labeling of the glaucoma and transection eyes and fellow eye controls was performed. In the normal monkey eye, TrkB labeling was somewhat heavier in the NFL and nerve head than in the myelinated optic nerve. Previous demonstrations of axonal transport interruption in monkeys with radiolabeled materials showed that IOP-induced blockade is associated with an increase in the labeling in the nerve head compared to the retina and the optic nerve.¹⁰ This pattern was present with the immunohistochemical techniques used here. Each monkey nerve head was placed into one of three categories: (1) normal pattern; (2) borderline (some relative increase in nerve head label); or (3) abnormal (definitely increased label in nerve head relative to retina and optic nerve). We also noted the presence of focal accumulations of TrkB and BDNF in the nerve head.

RESULTS

Normal Rat Optic Nerve Head Morphology

The normal rat optic nerve head shares many features with the primate (Figs. 1A, 1B). RGC axons organize into bundles as they pass from the retina into the optic nerve head. These bundles become lined by transversely oriented astrocytes and a rudimentary lamina cribrosa in the nerve head region. Approximately 1 to 1.5 mm behind the globe, the RGC fibers become myelinated and collectively are known as the optic nerve (Fig. 1B). Normal RGC axons when viewed by electron

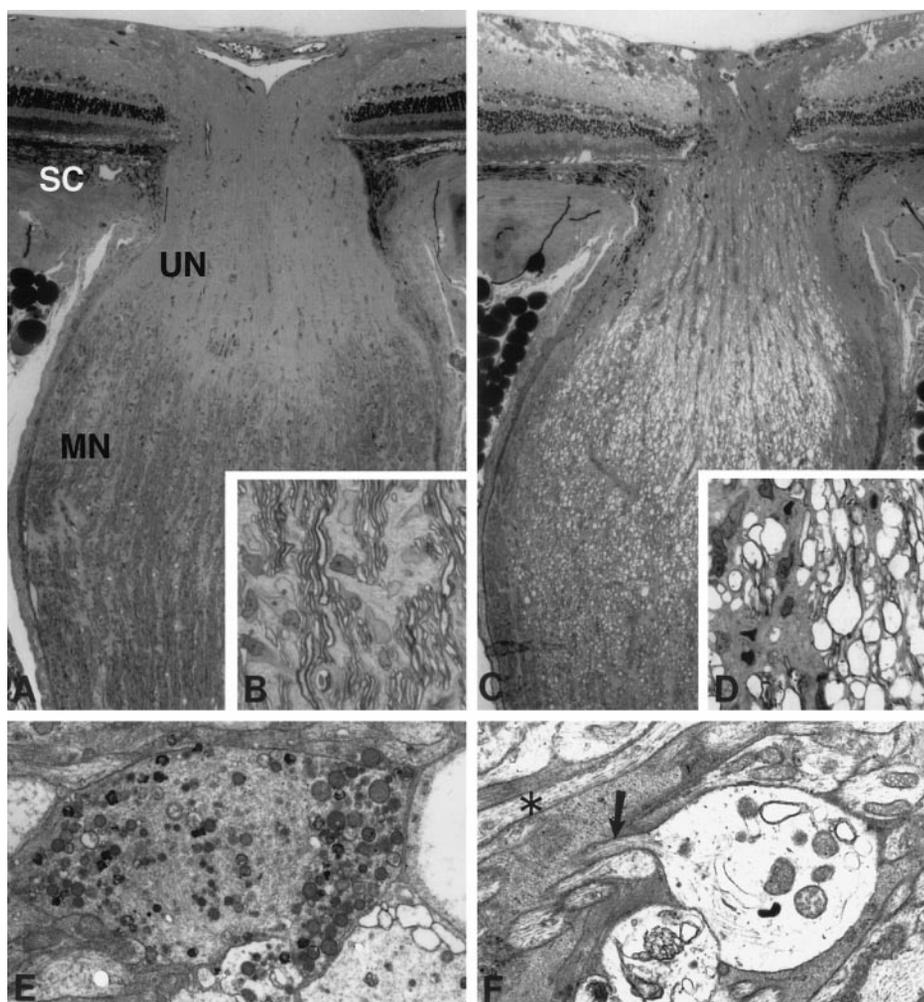


FIGURE 1. Morphology of rat optic nerve head at normal and elevated IOP. (A) Light micrograph of normal rat optic nerve head. Note that at the *top* the retina extends to either side of the scleral canal (SC) of the optic nerve head. Posterior to this canal, the optic nerve begins, first with an unmyelinated segment (UN), and then the myelinated optic nerve (MN) (toluidine blue stain; magnification, $\times 100$). (B) High-power view of normal axons from the myelinated optic nerve (toluidine blue stain; magnification, $\times 600$). (C) Light micrograph of elevated IOP nerve head shows clear, swollen axons behind the scleral canal and in the unmyelinated and myelinated optic nerve (toluidine blue stain; magnification, $\times 100$). (D) High-power view of axons appearing as clear areas at the junction between unmyelinated and myelinated nerve (toluidine blue stain; magnification, $\times 600$). (E) Electron micrograph demonstrating axons of the unmyelinated optic nerve swollen by accumulations of organelles including vesicles and mitochondria (magnification, $\times 8000$). (F) Clear zones within unmyelinated axonal bundles when viewed by electron microscopy are dilated nerve fibers. Note continuity of swollen, clear area with normal axon segment to its *left* (*arrow*). Organelles seen in swollen fiber include microtubules, mitochondria, and smooth vesicles. Compare to normal longitudinal axonal fiber (*asterisk*) in *upper left* (magnification, $\times 10,000$).

microscopy contain microtubules and neurofilaments. Other axonal elements include mitochondria and smooth vesicles.

Ultrastructural Evaluation of Rat Acute IOP Model

After elevation of eye pressure to produce PP = 25 mm Hg, some eyes demonstrated easily visible, clear areas among neural bundles in the unmyelinated nerve just outside the sclera (Figs. 1C, 1D). With transmission electron microscopy, there were abnormalities in axons of all eyes at PP = 25. The milder defects consisted of accumulations of vesicles within axons at the scleral opening and in the unmyelinated zone (Fig. 1E). The clear areas seen by light microscopy corresponded to widely

dilated RGC axons bounded by plasma membranes (Fig. 1F). Clumps of vesicles also were observed in axons proximal and distal to these swollen zones. In eyes with PP = 0, axon dilation also was observed at the level of the sclera and in the myelinated optic nerve. The severity of swelling and the degree of dilation was greater at the lower PP.

Trk and BDNF Labeling in Normal Rat Retina and Optic Nerve Head

In the normal rat, TrkB staining was found in the NFL, GCL, and inner plexiform layer, with the most intense labeling in the NFL (Fig. 2A). (Findings in the outer retina will be reported

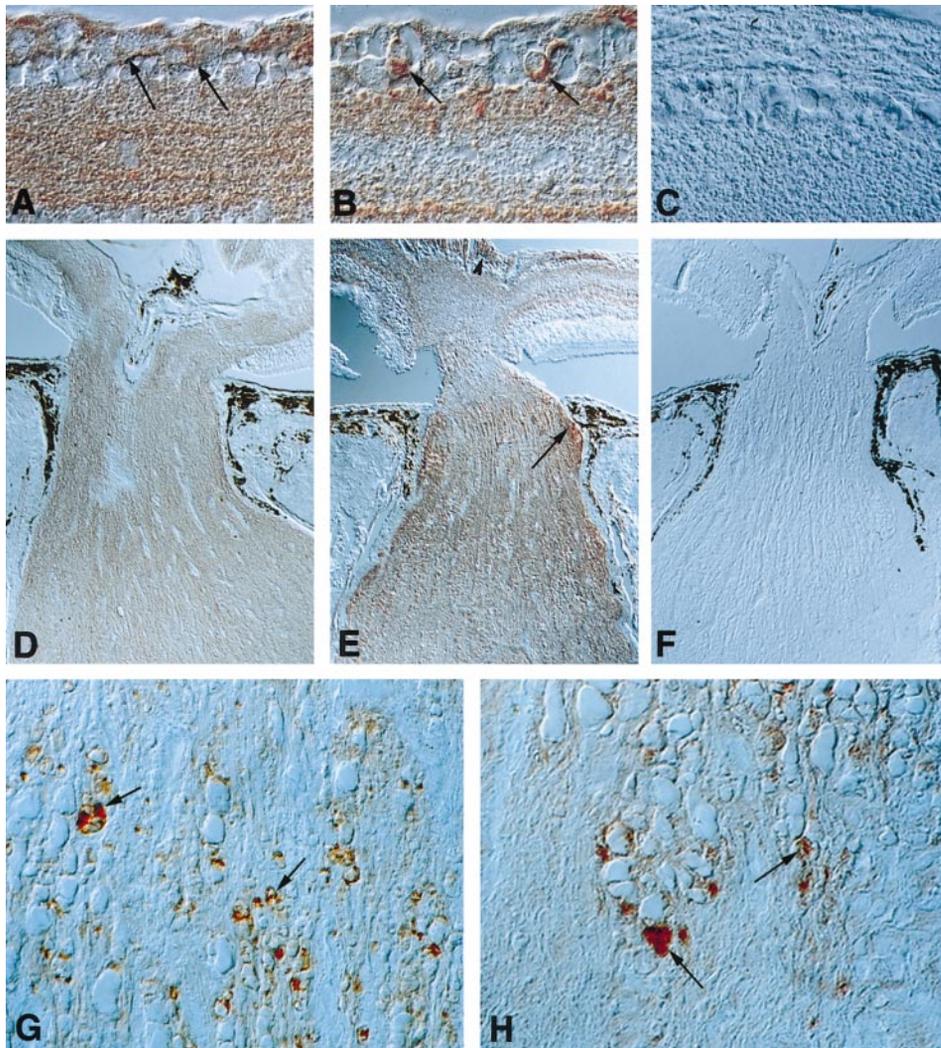


FIGURE 2. Immunolabeling of TrkB receptors in rat eyes. (A) TrkB in normal rat retina, with substantial label over NFL, GCL neurons (*arrows*) and the inner plexiform layer; (B) TrkB label in rat with 4-hour IOP elevation shows relatively more label over cytoplasm of GCL neurons (*arrows*) than in eyes with normal IOP; (C) retinal section incubated with TrkB antibody preabsorbed with excess antigen appears unstained, demonstrating the specificity of the antibody; (D) TrkB label over normal optic nerve head with uniform label from retinal NFL through nerve head to retrobulbar nerve (*below*); (E) elevated IOP produces increased TrkB label just outside the globe (*below*) compared to retinal NFL (*above*). Focal accumulations of TrkB label are seen in periphery of nerve head opposite choroid and sclera (*arrow*). Note increased NFL labeling after acute pressure elevation (*arrow-head*). (F) Optic nerve head incubated with TrkB antibody preabsorbed with excess antigen appears unstained, demonstrating the specificity of the antibody; (G) focal accumulations of TrkB label (*arrows*) in unmyelinated RGC axons from rat with elevated IOP; (H) focal accumulations also were observed in areas of dilated myelinated axons (*arrows*). Magnification, (A through C, H) $\times 535$; (D through F) $\times 85$; (G) $\times 615$.

separately by McKinnon et al.) TrkB label was uniformly distributed in the neural bundles of the optic nerve head and optic nerve (Fig. 2D). Glial cells of the nerve head also were labeled, but at an intensity less than that of the axonal fibers. There was no labeling in slides with primary antibody blocked with excess control peptide (Figs. 2C, 2F), nor in slides stained with nonimmune IgG or incubation buffer (data not shown).

TrkA was found in the same layers of the rat retina as TrkB, although it labeled the NFL, GCL, and inner plexiform layer less intensely than TrkB (data not shown). BDNF labeling was barely detectable, and TrkC immunoreactivity was undetectable in the rat retina even with the optimal protocol (data not shown).

TrkB Localization with Acute IOP Elevation in Rats

In five of six PP = 0 eyes, TrkB labeling was more intense than in normal retinas in the NFL and GCL (Fig. 2B). In the optic nerve head, focal accumulations of antibody label were identified in neural bundles passing through the nerve head (Fig. 2E). Similar accumulations also were found in the unmyelinated optic nerve and occasionally among myelinated axons (Figs. 2G, 2H). In areas with focal labeling, both dilated and intact axons were densely labeled for TrkB. Eyes at PP = 25 had less intense accumulation of TrkB label than that of eyes at

PP = 0 at the same antibody concentration. The PP = 0 eyes had a statistically higher mean severity score (2.2) than the PP = 25 eyes (mean = 2.0) or the normal eyes (mean = 0.8). The IOP ranges were 51 to 81 mm Hg for the PP = 0 group and 19 to 58 mm Hg for the PP = 25 group (Table 1). In two eyes noted to have some leakage at the corneal needle, the IOP appeared to be above the normal range during the experiment. We did monitor the IOP levels by Tonopen XL tonometry and did not confirm that these eyes failed to achieve the desired IOP. However, it was interesting to us that their TrkB results were closer to eyes with normal IOP than to the other eyes with elevated IOP.

In nine rat eyes with elevated IOP, we could examine a section of the entire nerve head, retina, and optic nerve on the same slide to compare the labeling in each area. In seven of these nine, TrkB label was greater in the optic nerve than in the nerve head or retina (Fig. 2E). In addition, cells presumed to be astrocytes that line the nerve bundles were intensely stained after IOP elevation, especially in the nerve head. The masked grader (HAQ) correctly identified 8 of 11 IOP elevation eyes and 6 of 11 control nerve heads based on their TrkB label patterns. Severity scores in elevated IOP eyes were significantly different from their fellow, control eyes (paired *t*-test, $P = 0.007$; Table 1).

TABLE 1. Masked Grading of TrkB Labeling in Rats with Acute Glaucoma

RAT ID	IOP	BP	Intended Perfusion Pressure	Actual Perfusion Pressure	Severity Score	Mean Group Score
1R	51	51	0*	0	2	2.2
3R	62	62	0	0	3	
2R	65	65	0	0	3	
7R	71	71	0	0	2	
6R	81	81	0	0	3	
8R	77	77	0*	0	0	
5R	19	44	25	25	3	—
4R	25	50	25	25	3	2.0
11R	47	72	25	25	3	
10R	48	73	25*	25	0	
9R	58	83	25*	25	1	
5L	17	44	C	27	1	—
4L	16	50	C	34	1	1.6
1L	13	51	C	38	2	
3L	15	62	C	47	2	
2L	17	65	C	48	2	
10L	21	73	C	52	0	—
7L	16	71	C	55	0	0.2
11L	15	72	C	57	0	
8L	15	77	C	62	0	
6L	14	81	C	67	0	
9L	16	83	C	67	1	

IOP = intraocular pressure (mm Hg), BP = blood pressure (mm Hg).

* These eyes had leakage around the corneal cannula that was controlling IOP.

Severity scores of experimental and their fellow, control IOP eyes were significantly different (paired *t*-test, $P = 0.007$, $t = 3.357$, $df = 10$).

Two interesting results occurred as byproducts of the experimental system. First, some animals had relatively low BP during the 4-hour experiment, causing the actual PP in control eyes with lower BP to be close to that in PP = 25 rats with normal BP. To assess the effect of this factor, the actual PP of the experimental and control eyes was compared to their severity score (Table 1). Abnormality in TrkB labeling closely followed actual PP, with the majority of eyes less than 30 mm Hg having severity scores of 2 or 3, and those with higher PP usually scoring 0 or 1. Another link between PP and transport

block was the fact that four of the five controls that were graded as abnormal in TrkB labeling had low BP throughout the experiment. With controls divided into those whose PP was between 25 and 50 mm Hg and those higher than 50 mm Hg, the mean severity scores of the groups were 1.6 and 0.2, respectively (Table 1). In a few eyes, there was detectable leakage around the corneal cannula, leading to a failure to achieve the desired IOP for some portion of the experiment. Consistent with this interpretation, two of four experimental eyes with a normal TrkB label pattern by masked grading were noted during the time of surgery to have leakage of fluid at the corneal cannula (Table 1).

TrkA and BDNF localization in the retina, nerve head, and optic nerve was not detectably altered by acute IOP elevation in areas occupied by RGC and their axons (data not shown).

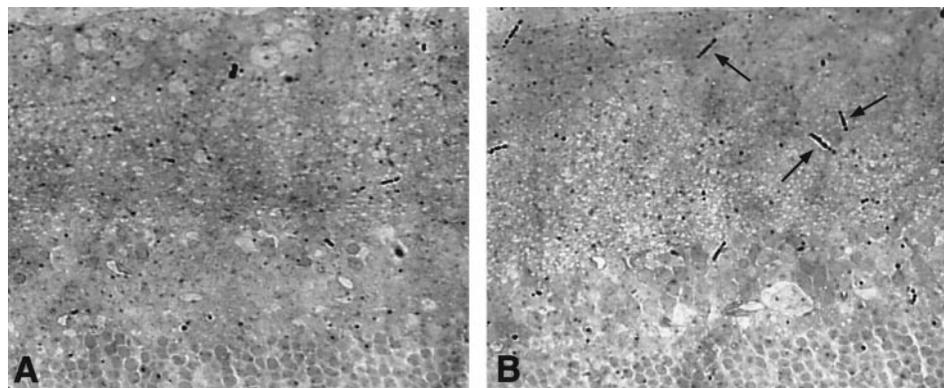
Autoradiography of ^{125}I -BDNF

The eight eyes with IOP elevation had a 60% reduction in transported radioactivity overlying the inner retina (48.8 ± 41.9 grain tracks/ μm^2) compared to the mean of control eyes that also had bilateral colliculus injections of ^{125}I -BDNF (121.8 ± 22.1 grain tracks/ μm^2 , $P = 0.002$) (Fig. 3). The values for the PP = 25 and the PP = 0 eyes were both depressed to a similar degree. In the two animals in which competitive inhibition was tested by unilaterally injecting a mixture of 100-fold excess unlabeled BDNF with ^{125}I -BDNF into the superior colliculus, there was a 37% decrease in radioactivity arriving at the retina (60.8 ± 15.8 compared to 96.8 ± 46.2 grain tracks/ μm^2 for matched control animals that had unilateral collicular injection of ^{125}I -BDNF). Coinjection of cold NGF with ^{125}I -BDNF in two animals did not cause any decrease in transport (94.2 ± 19.4 compared to 96.8 ± 46.2 grain tracks/ μm^2 for the control eyes).

Normal Monkey Findings with TrkB and BDNF

Retinal ganglion cells from normal monkey retinas demonstrated TrkB label (Fig. 4A). In the normal monkey optic nerve head, TrkB labeling was more intense in the nerve head compared to the myelinated portion of the optic nerve, possibly because of the presence of the myelin sheaths in the nerve that decrease the proportion of the tissue comprised of axons (Fig. 4B). Astrocytes and oligodendrocytes also labeled positively for TrkB. BDNF labeling was uniformly present in nerve head and optic nerve of normal monkey eyes. Control slides incubated

FIGURE 3. Autoradiography of ^{125}I -BDNF in elevated and normal IOP rat retina. Note the relative lack of grain tracks in the elevated IOP inner retina (A) compared to the normal IOP inner retina (B) (arrows). Magnification, $\times 300$



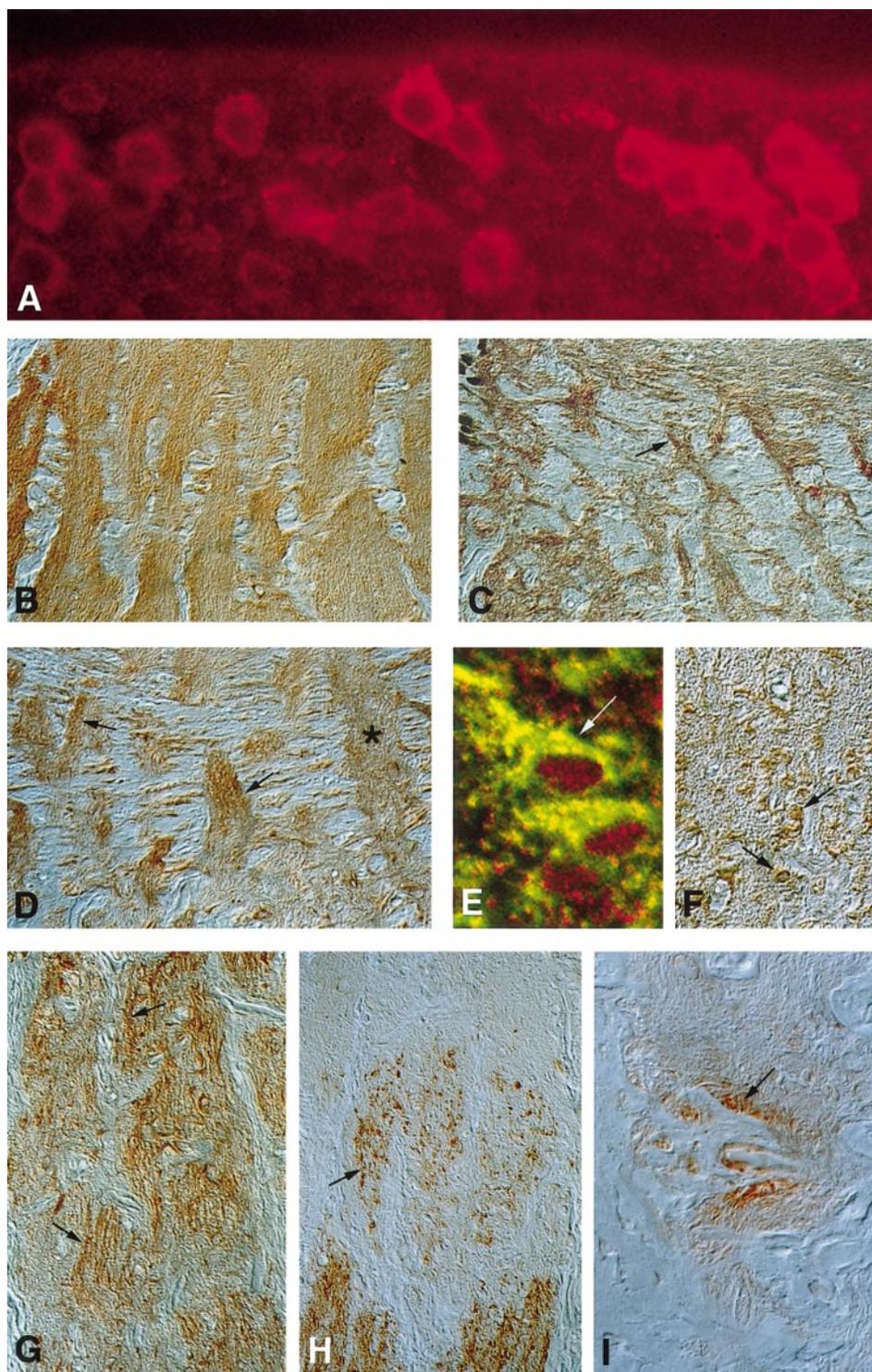


FIGURE 4. Monkey optic nerve heads labeled for TrkB (A through D, F, G), TrkB and GFAP (E), and BDNF (H, I). (A) Retinal ganglion cells in normal monkey inner retina are labeled with TrkB, appearing red by epifluorescence. (B) Normal monkey at nerve head, illustrating uniform labeling of TrkB in nerve bundles and unlabeled optic nerve head connective tissue. (C) After complete optic atrophy from nerve transection, the relative area occupied by nerve head connective tissue is increased and zones formerly occupied by neural bundles are severely thinned. Remaining TrkB label is seen in areas known to be filled in by astrocytes after transection (arrow). (D) TrkB focal accumulations (arrows) in neural bundles of nerve head from monkey with chronic, moderate glaucoma damage. Compare to uniformly stained neural bundle on right (asterisk). (E) TrkB (pseudocolored red) and GFAP (pseudocolored green) are colocalized in astrocytes (arrow) in the formerly myelinated optic nerve. Where overlap of TrkB and GFAP labeling occurs, a yellow color is produced. Such prominent labeling for TrkB in astrocytes was detected after both transection (as in this example) and after chronic glaucoma, but not in normal eyes. (F) Residual TrkB labeling remains over glial cells (arrow) after complete atrophy from optic nerve transection. (G) In monkey eye with moderate glaucoma damage, myelinated axons show focal accumulations of TrkB label (arrows). (H) Changes in BDNF label are demonstrated in this monkey eye with mild glaucoma. Punctate label is observed in axons near the nerve head (arrow) in contrast to the uniform label in the myelinated nerve (below) and in normal eyes (not shown). (I) Focal accumulation of BDNF label (arrow) is observed in a neural bundle immediately adjacent to nerve head connective tissue in this monkey eye with moderate glaucoma damage. Magnification, (A) $\times 875$; (B through D, F through H) $\times 240$; (E) $\times 1250$; (I) $\times 535$.

with antibody blocked by excess control peptide or nonimmune IgG were negative (data not shown). Antibodies against TrkA and TrkC were not studied in monkeys.

Glaucomatous Monkey Findings

Of six monkeys with experimental glaucoma, two eyes each had mild, moderate, and severe damage (Table 2). The glaucoma optic nerve heads demonstrated one or more of three

alterations in TrkB labeling (Figs. 4D, 4F, 4G): (1) a relative increase in the axonal labeling in the nerve head compared to the superficial retina and the myelinated nerve (Fig. 4D); (2) focal, intensely labeled fibers in the nerve head (Fig. 4G); or (3) prominent glial labeling that was not detected in normal eyes (demonstrated in Fig. 4F in an astrocyte from a transection eye). BDNF labeling showed similar focal accumulations in the same areas as TrkB (Figs. 4H, 4I).

TABLE 2. Summary of Monkey Clinical Data

Monkey ID	Mean IOP (mm Hg)	Duration of IOP Elevation	IOP Just Prior to Death (mm Hg)	Estimated Axon Loss (%)	Grading of Clinical Injury
Glaucoma					
M950R	32	2 years	34	10	Mild
M970R	43	4 months	45	<10	Mild
M971R	26	7 months	27	25	Moderate
M965R	33	4 months	47	20	Moderate
M957R	25	7 months	19	>75	Severe
M968R	39	6 months	56	60	Severe
Transection					
M966R	N/A	N/A	N/A	100	Time After Transection
M967R	N/A	N/A	N/A	100	5 months

Normal IOP mean = 19 mm Hg; N/A, not applicable.

Optic Nerve Axotomy Findings

In two eyes with complete atrophy after optic nerve axotomy, there was a significant decrease in axonal label for TrkB and BDNF (Figs. 4C, 4F). However, TrkB labeling was found on the margins of connective tissue beams in the nerve head and in atrophic zones of the retrolaminar nerve, apparently associated with glia. Double labeling of TrkB and GFAP demonstrated colocalization within astrocytes of the axotomized optic nerve head (Fig. 4E).

Quantification of Masked Evaluation of TrkB Labeling in Monkey Models

Eight control, six glaucoma, and two transection nerve heads were graded by a masked observer. Seven of eight controls were graded normal, and the eighth was graded as borderline (Tables 3, 4). Five of the six experimental glaucoma nerve heads had increased nerve head accumulation of TrkB. The sixth glaucoma nerve head had severe atrophy, eliminating any RGC label. Focal TrkB accumulation was seen in five of six glaucoma eyes, but in only one of eight controls. The differences between control and glaucoma eyes were significant ($P = 0.026$, Fisher's exact test; Table 4). Focal TrkB accumulation observed in transection eyes was associated with glia and axons were uniformly absent (Fig. 4F). Because of the limited number of monkey sections available, quantification of the findings from the masked evaluation of BDNF labeling was not performed.

DISCUSSION

Our study confirms the presence of TrkB receptors and BDNF in the GCL and optic nerve of the rat.^{23,28} To our knowledge,

TABLE 3. TrkB Labeling in the Optic Nerve Heads of Monkeys with Chronic Glaucoma and Nerve Transection

	Normal	Borderline	Abnormal
Normal ($n = 8$)	7	1	0
Glaucoma ($n = 6$)	0	0	5
Transection ($n = 2$)	0	0	2

One chronic glaucoma eye (not given a grade in the Table) was noted to have such severe atrophy that it could only be graded as having no specific axonal labeling. Borderline, questionable increase in nerve head label relative to retina and optic nerve; abnormal, definite increase in label of nerve head compared to retina and optic nerve.

this is the first evidence for expression of TrkB and BDNF by primate RGCs. Full-length TrkB receptor was previously found to predominate in the adult rat retina with northern blot analysis and in situ hybridization, whereas the truncated tyrosine kinase receptor type predominated in the optic nerve.^{23,26,41} The adult avian GCL also produces mRNA for BDNF and TrkB.^{29,30} Glial involvement in the production of TrkB receptors has been reported in cultured types 1 and 2 astroglia, microglia, and oligodendrocytes from the optic nerve, which express both truncated and full-length TrkB receptors, with the truncated form predominating.⁴¹ Our axotomy experiments provide clear evidence that TrkB is present in RGC axons, because labeling over neural bundles disappeared after fiber degeneration, except that associated with glial cells.

Our data suggest that TrkB receptors moving in axonal transport within optic nerve fibers are blocked at the optic nerve head in rat and monkey eyes in glaucoma models. Acute IOP elevation causes disruption of axonal transport in RGCs, indicated by accumulation of smooth-surfaced vesicles within axons, a finding that is similar to observations after chronic IOP elevations in rat eyes.⁴² Such vesicle collections colocalize with blocked radioactive protein moving in anterograde axonal transport after acute IOP elevation in the monkey.^{9,10,12,13} Retrograde transport of horseradish peroxidase was found to be obstructed at the site of such vesicular accumulation with short-term IOP elevation in monkey eyes.¹¹ Acute IOP elevation has been previously shown to obstruct retrograde axonal transport in rat eyes.⁴³ The dramatic swelling of axons just behind the sclera in the rat optic nerve has not been described after acute IOP increase in monkey eyes. In monkeys, axons are myelinated immediately behind the nerve head, perhaps preventing the swelling observed in these nonmyelinated segments of rat axons. The frequent association of these clear

TABLE 4. Presence of Focal TrkB Accumulation in Monkey Optic Nerve Heads

	Present*	Absent	P Value†
Normal ($n = 8$)	1	7	
Glaucoma ($n = 6$)	5	1	0.026
Transection ($n = 2$)	2	0	

* Present refers to significant focal increase in TrkB label within 1 or more neural bundles within the lamina cribrosa.

† Proportion of normal and glaucoma eyes with focal TrkB label is significantly different (Fisher's exact test).

areas with accumulations of intracellular vesicles and mitochondria suggests transport obstruction.

The immunohistochemical findings provide further evidence for transport obstruction that affected the distribution of BDNF and TrkB in experimental glaucoma. The normal pattern of TrkB labeling was altered in four important ways in both rat (acute model) and monkey eyes (chronic model). These were (1) alteration of normal TrkB axonal distribution, (2) focal accumulations of TrkB and BDNF, (3) increased label in GCL neurons for TrkB, and (4) increased TrkB label in glia.

In seven of nine rat eyes at PP \leq 25 mm Hg, TrkB label was more apparent in the optic nerve than in the retina. Because the degree of labeling varies from slide to slide, we depended on masked comparison within each slide of intraretinal, nerve head, and optic nerve label to make reproducible, valid judgments. Relative increase in axonal labeling behind the nerve head compared to labeling within the eye indicates obstruction of the retrograde TrkB receptor movement. In monkeys with chronic glaucoma, TrkB labeling increased in the nerve head compared to the retina or optic nerve, and BDNF and TrkB accumulated focally in the nerve head. In addition, acute IOP elevation in the rat decreased retrograde transport of radioactive BDNF. In eyes with PP = 0, the IOP level clearly impaired retinal blood flow, but in PP = 25 eyes, blood flow was normal, yet IOP elevation blocked BDNF retrograde movement.

We did not demonstrate labeling for BDNF either normally or with acute IOP elevation in rats, though there was focal BDNF accumulation by immunohistochemistry in chronic monkey glaucoma eyes. This may be due to species differences in the specificity of the antibody to human BDNF used here.

TrkB receptors have been shown to be internalized after ligand binding and move to the cell body by retrograde transport in activated form within smooth-surfaced vesicles.^{14-17,44} We observed the accumulation of such vesicles in both the chronic glaucoma model in monkeys¹² and in acute rat model eyes by electron microscopy. The intensely stained, focal collections of TrkB and BDNF label probably represent activated TrkB receptor/BDNF complexes at the nerve head level. It would be logical that significant IOP elevation would lead to a generalized breakdown in transport of many molecules. Yet, although we found TrkA to be present in axons, we found no change in distribution of this receptor. Either our methods did not produce sufficient labeling to observe transport obstruction, or the blockade affects TrkB more than TrkA.

There was a relative increase in TrkB labeling of GCL cell bodies in both rats with acute IOP elevation and in the chronic monkey model. In normal rat eyes, there was minimal label overlying cells in the GCL at antibody concentrations that intensely label axons. With IOP elevation, the neuronal cytoplasm was more darkly labeled than axons by TrkB antibodies. This could result from decreased receptor proteolysis, a failure of synthesized receptor to leave the cell body, increased production of receptor, or increased arrival of receptors from the axon-terminal. Increased arrival is made unlikely by the evidence for blockade of retrograde transport. Immunohistochemical methods cannot measure increased receptor synthesis. Such a response might be an interesting feedback response to lower levels of activated TrkB caused by the blockade of retrograde transport from experimental glaucoma.

The increased TrkB label over glial cells in the nerve head and optic nerve with experimental glaucoma and transection

appeared to be associated with astrocytes, as shown by cells double-labeled for TrkB and GFAP. In the retrobulbar nerve, increased glial labeling might involve oligodendrocytes, astrocytes, or microglia, perhaps by increased production of TrkB by glia, which normally synthesize both full-length and truncated TrkB.⁴⁵⁻⁴⁸ The antibodies used here recognize the full-length TrkB. Alternatively, glia could have acquired more receptor by phagocytosing axonal debris as neurodegeneration occurred.^{12,49}

Two technical issues in the acute rat model are worthy of comment. Leakage around the anterior chamber cannula was detected in 4 of 11 rats. Three of these four failed to show alteration in TrkB labeling. In subsequent eyes without visible leakage, abnormal TrkB labeling was present on masked evaluation in each case. Secondly, 5 rat eyes at normal IOP had increased labeling in the retina compared to the optic nerve, instead of the normal, uniform TrkB label seen in 6 other controls. These 5 animals had mean BP of 54 ± 8.8 mm Hg compared to 76 ± 5.0 mm Hg in the other controls (*t*-test, *P* = 0.000). Excessive pentobarbital anesthesia may have lowered BP in these animals, and this was corrected by lower doses in later animals. The control eyes with lower BP therefore had PP intermediate between eyes with high IOP and the other controls (mean actual PP = 38.3 ± 8.9 mm Hg, compared to mean of other 6 controls = 60.0 ± 6.3 mm Hg; *t*-test, *P* = 0.001). This probably explains their intermediate levels of abnormality in TrkB labeling.

Although a masked grader evaluated rat and monkey tissues, severe chronic glaucoma injury and axotomy produce alterations that are clearly visible to the experienced observer. However, in two monkey eyes with mild optic nerve fiber loss and no histologically visible atrophy, there was relative increase and focal accumulation of TrkB in the nerve head.

The additional experiments with acute IOP elevation in rats after ¹²⁵I-BDNF injection into the superior colliculus show that movement of axonally transported neurotrophin is substantially inhibited by increased IOP. Furthermore, the data with coinjection of nonradioactive NGF and BDNF show that only the latter affects the arrival of ¹²⁵I-BDNF in the retina. This strongly supports the concept that the movement of BDNF is being mediated by the TrkB receptor. Hence, our studies of movement of the TrkB receptor are made more relevant to delivery of BDNF under normal conditions in the adult rat eye and to inhibition of neurotrophin delivery in experimental glaucoma.

Our interest in TrkB receptors derived from the fact that its ligand BDNF is known to be an important trophic factor for RGCs. BDNF supports the survival of retinal explants and cultures, inhibits apoptosis, and increases the rate of axonal elongation.^{19,20,24,50,51} Rat RGCs have been reported to demonstrate a modestly extended life after axotomy when BDNF is injected intravitreally.^{22,24,27,31,32,52} Our experiments suggest that signaling from one endogenous source of BDNF, target cells in the superior colliculus (rat), and lateral geniculate (monkey) may be interrupted in glaucoma, thereby affecting the influence this pathway has on RGC survival. These results support the hypothesis that trophic actions of tyrosine kinase receptor-mediated mechanisms on RGCs may be an important component of the pathway from axonal injury to RGC death.

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