

Retrograde Axonal Transport of BDNF in Retinal Ganglion Cells Is Blocked by Acute IOP Elevation in Rats

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PURPOSE. To determine whether acute experimental glaucoma in rats obstructs retrograde transport of brain-derived neurotrophic factor (BDNF) to retinal ganglion cells (RGCs).

METHODS. Forty rats had unilateral injection of either ^{125}I -BDNF (20 animals) or a mixture of ^{125}I -BDNF and 100-fold excess nonradiolabeled BDNF (20 animals). In each group of 20 animals, eyes contralateral to injection had either normal intraocular pressure (IOP; 10 animals) or IOP elevated to 25 mm Hg below the systolic blood pressure of the eye (10 animals). In each group of 20 rats, ipsilateral eyes had IOP set at systolic blood pressure (4 eyes), had optic nerve transection (10 eyes), or had normal IOP (6 eyes). Six hours after injection, animals were killed and tissues were fixed, embedded, and sectioned for autoradiography. Grain counts were performed over retina and optic nerve using automated image analysis.

RESULTS. IOP elevation to 25 mm Hg below systolic blood pressure (perfusion pressure [PP] 25) decreased median retinal nerve fiber layer (NFL) grains by 38% compared with controls ($P < 0.001$). Competition by cold BDNF reduced NFL grains by 28% ($P = 0.013$). Considering only the radioactivity representing specific retrograde transport of BDNF, IOP elevation to PP25 reduced transport by 74%, whereas elevation to PP0 (equaling systolic blood pressure) reduced specific transport by 83%.

CONCLUSIONS. BDNF is transported retrogradely from the superior colliculus in adult rats, and this transport is substantially inhibited by acute IOP elevation. Deprivation of BDNF among RGCs may contribute to neuron loss in glaucoma. (*Invest Ophthalmol Vis Sci.* 2000;41:3460-3466)

The events of retinal ganglion cell (RGC) death in glaucoma have been studied in increasing detail. It is recognized that mammalian RGCs whose axons are cut or severely injured along the nerve or tract generally do not survive. In 1977, we proposed that the death of monkey RGCs after orbital optic nerve transection resulted from the failure of retrograde axonal transport to provide important, but unknown, factors to the cell body.¹ Anderson and Hendrickson² have shown that experimental glaucoma obstructs orthograde axonal transport in monkeys and similar obstruction of retrograde transport in experimental monkey models,³ as well as blockade in human glaucoma eyes, has been demonstrated.⁴ These findings suggest that the interruption of the supply of an

unknown factor to RGCs may contribute to cell loss in glaucoma.

Candidates for important trophic factors among RGCs now include a variety of growth factors and neurotrophic factors. One such molecule that appears to be of particular importance to RGCs is BDNF. RGCs are produced in excess in fetal life, then pruned to only those properly targeted to central nervous system centers. Target-derived stimulation by trophic factors, including BDNF for RGCs, has been shown to promote neuronal survival.⁵⁻¹⁰ BDNF, in particular, has been shown to increase survival of photoreceptors in inherited retinal degeneration, light toxicity, and experimental retinal detachment.¹¹⁻¹³ BDNF also retards RGCs and inner retinal neuronal loss after retinal hypoxia and optic nerve transection.¹⁴⁻¹⁶ BDNF and associated neurotrophins interact with specific transmembrane cell surface receptors that dimerize after binding of the ligand and are incorporated into vesicles for retrograde transport to the cell body.¹⁷

Acute and chronic models in the rat have begun to contribute to investigations in glaucoma research.¹⁸⁻²⁰ The production of elevated IOP in rats has been shown to lead to RGC death. The relatively lower cost and convenience of the rat for studies of new glaucoma therapies recommends such models by comparison to primate models. In a previous report, we demonstrated that the primary BDNF receptor TrkB is present in optic nerve axons of monkey and rat and that its distribution changes with acute and chronic experimental glaucoma in a

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manner suggesting transport interruption at the optic nerve head region.²¹ In a small number of eyes in that study, we measured radioactive BDNF movement after intracranial injection, corroborating the probable functional significance of TrkB obstruction. The present report was designed to study in detail the retrograde transport of BDNF in the adult rat, to differentiate specific axonally transported BDNF from nonspecific movement of BDNF into the retina after intracollicular injection, and to quantify in greater detail the degree of blockade of BDNF movement by experimental IOP elevation.

METHODS

Forty adult male brown Norway rats (*Rattus norvegicus*), weighing approximately 250 g, underwent unilateral injections into the superior colliculus by means of a stereotaxic apparatus. Coordinates were determined using adjusted coordinates from a published atlas,²² with corrections for size based on the measured distance between the cranial sutures, lambda to bregma, of each rat. Rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg; Sigma). All procedures abided by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty animals received a 3- μ l injection of ¹²⁵I-BDNF at a specific activity of 1 μ Ci/ μ l (hot BDNF group). The biologic activity of an aliquot of this radioactive BDNF was confirmed in vitro in an assay, by using dorsal root ganglion cells.²¹ Twenty additional rats received one 3- μ l injection containing the same amount of ¹²⁵I-BDNF mixed with 100 molar excess nonradiolabeled BDNF (cold competition group). The side of the brain to be injected was chosen randomly. The majority of optic nerve fibers cross to the opposite side of the brain in the rat, with the actual proportion that cross exceeding 90% in most studies.

In 10 rats each in the hot BDNF and the cold competition groups, the eye contralateral to collicular injection had normal IOP for 6 hours after injection. IOP was monitored (Tonopen XL; Mentor, Norwell, MA) with topical proparacaine eyedrop anesthesia. In 10 additional rats in each group, the contralateral eye was set at IOP equal to 25 mm Hg below the systolic ocular blood pressure. To accomplish this, we placed a blunt 30-gauge needle into the anterior chamber, connected to a variable-height reservoir of saline. Systolic blood pressure was estimated by raising the reservoir until flow in the central retinal artery was shown to cease by indirect ophthalmoscopy. In extensive comparisons, we raised and lowered IOP using the reservoir and anterior chamber needle, while monitoring the femoral artery blood pressure from a cannula connected to a transducer. The ophthalmoscopic estimation of systolic and diastolic blood pressure in the retinal arteries was reproducibly 10 mm Hg below that obtained in the femoral artery of several rats with different blood pressures under anesthesia. Brief rechecks of the systolic retinal artery pressure level were made during the experiment. We estimate that flow to the eye was affected for less than 1 minute by the ophthalmoscopic procedure for each rat. The level of anesthesia was assessed by noting the respiration rate and response to paw stimulation, and supplemental pentobarbital sodium was administered to maintain stable anesthesia. At 6 hours after collicular injection (and IOP elevation in half of the eyes), animals were killed with pentobarbital and the eyes rapidly enucleated.

To investigate potential nonaxonal sources of BDNF movement, four protocols were performed with the eyes ipsilateral

to collicular injection. These were: 1) To eliminate any axonal transport contribution, the ipsilateral optic nerve was transected in eight eyes (without suture ligation); 2) to prevent not only axonal transport, but also diffusion through cerebrospinal fluid through cut ends of the nerve sheath, the ipsilateral optic nerve was transected, and the distal optic nerve and sheath were ligated with a 10-0 nylon suture (12 eyes); 3) to prevent axonal transport and to stop entry of BDNF through the vasculature, IOP was elevated in ipsilateral eyes to a level equal to blood pressure (8 eyes); and 4) 12 ipsilateral eyes were maintained at normal IOP as controls. In each of the four groups, half of the animals received only ¹²⁵I-BDNF (hot BDNF) in their unilateral collicular injection and half were injected collicularly with the mixture of cold and radioactive BDNF (cold competition). Optic nerve transections were performed before collicular injection.

Tissues were fixed by immersion in 2% paraformaldehyde-2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in epoxy resin, and 1- μ m-thick sections were placed on glass slides. Sections were used that included the optic nerve head, optic nerve, and nasal and temporal retina. Epoxy sections were dipped in liquid emulsion (NTB-2; Scientific Imaging Systems, Eastman Kodak, Rochester NY). After 2-month exposures, these were developed, and the number of grains per square millimeter was counted with an image analysis instrument (Vidas; Carl Zeiss, Thornwood, NY). In one slide from each eye, retinal counts were taken from six areas of the nerve fiber layer, from two areas of the photoreceptor layer (as background estimates), from the optic nerve head, and from the unmyelinated and myelinated optic nerve. The median values for each retina were calculated and are reported here rather than mean values. This was done because the data from some groups of eyes were not normally distributed and the median value better expressed the group behavior. One observer performed all counting and was masked to the protocol used for the eyes.

RESULTS

In three contralateral eyes and two ipsilateral eyes, tissue preservation or sectioning was inadequate to interpret data. In one animal, there was no radioactivity above background in the retina of either eye, presumably due to a collicular injection that was misplaced. As a result, there were useful data for 36 (90%) of the 40 contralateral eyes and 37 (92.5%) of the 40 ipsilateral eyes that began the experiment.

We chose to estimate BDNF transport by measuring the grain density over the nerve fiber layer (NFL). Although transport to RGC bodies was also of interest, counting over the layer with RGC bodies added large variations due to the presence of the unlabeled RGC nuclei (Fig. 1). This was most apparent in comparing counts in the most posterior retina where RGC body density was highest with counts in more peripheral retinal zones. Qualitative examination of the position of autoradiographic grains overlying the tissue showed a consistent pattern. Grains were dense over the retinal NFL and in the cytoplasm of RGCs, but not over their nuclei. The inner plexiform layer had fewer overlying grains than the NFL, and the remainder of the retina had almost none (Fig. 1). The 36 contralateral eyes had a median of 738 grains/10,000 μ m² over the NFL and 9 grains/10,000 μ m² over photoreceptors. We

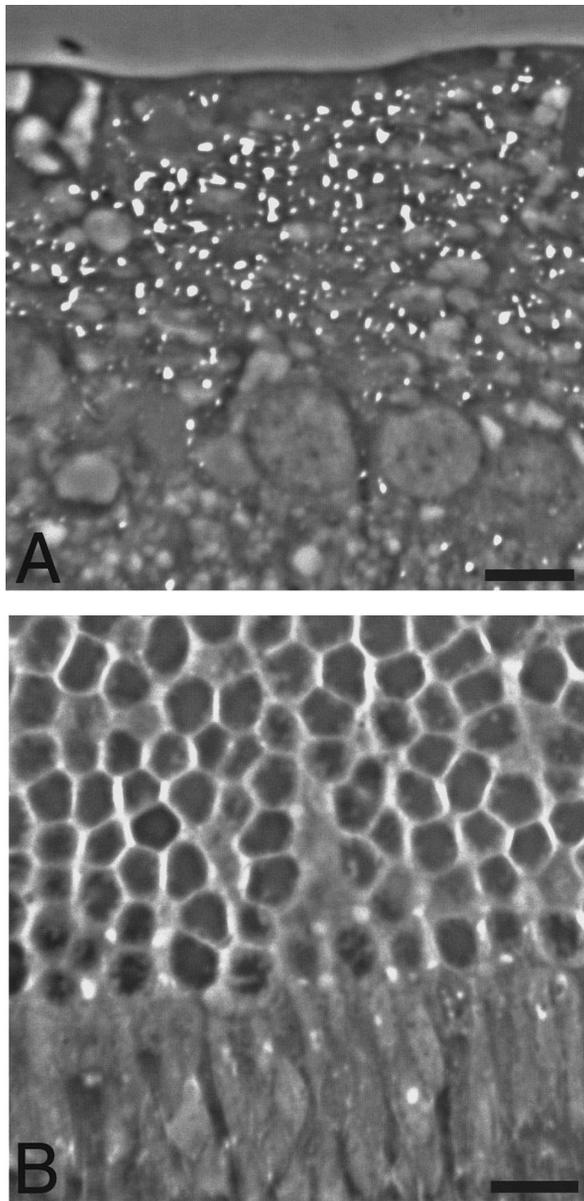


FIGURE 1. Rat retina 6 hours after intracollicular injection of radiolabeled BDNF in an eye contralateral to injection that was kept at normal IOP. Autoradiographic grains in this view appear as white dots. (A) There was high grain density over the axons in the nerve fiber layer and over the RGC cytoplasm but not the nuclei. Many fewer grains were over the inner plexiform layer (A; *bottom*). (B) No grains were visible overlying the photoreceptor layer, indicating a low level of background label compared with BDNF transport to RGCs. Phase contrast; bar, 50 μm .

considered the latter to be an estimate of background radioactivity—approximately 1.3% of the grain density in the area of most interest. There was a dramatic decrease in grain density from the superficial NFL inside the eye into the optic nerve head, where axonal bundles had far lower densities than in the nearby retina (Fig. 2). For example, for all contralateral eyes ($n = 36$) the optic nerve head at the level of the choroid had a median grain density of 15 per 10,000 μm^2 or 2.1% of that in the nearby NFL.

IOP elevation caused a substantial decrease in retinal grain density (Table 1). For those contralateral eyes with IOP in-

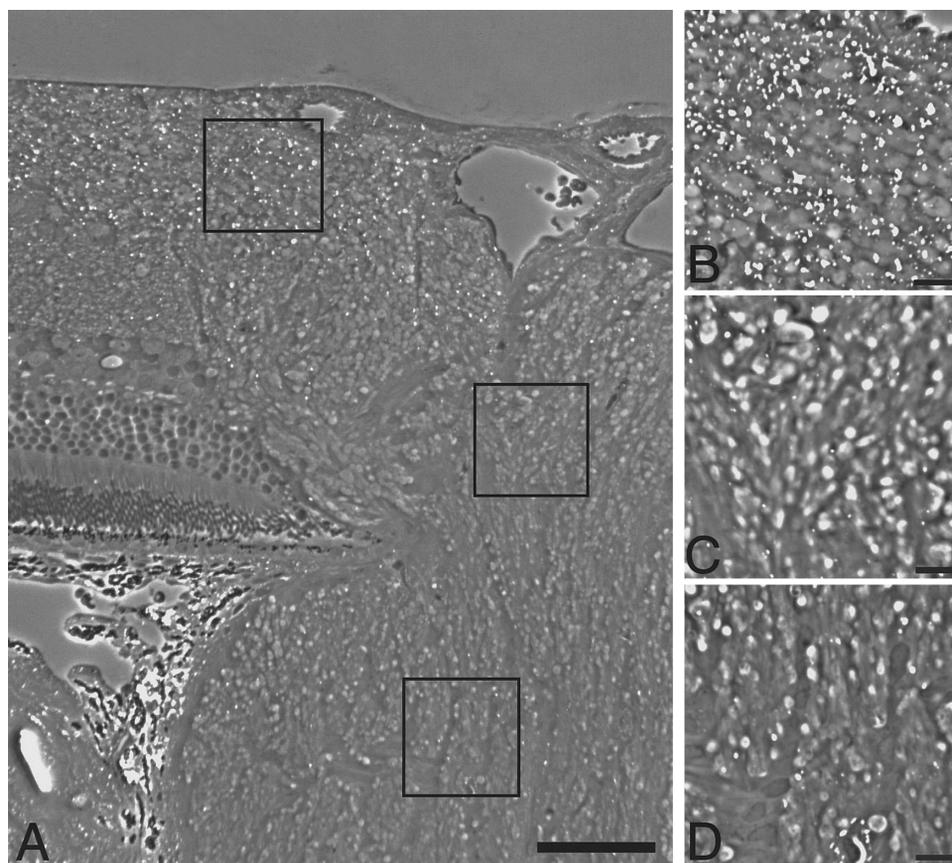
creased to 25 mm Hg below systolic blood pressure (PP25), the overall reduction in median grain density was 38% compared with contralateral eyes at normal IOP ($P < 0.001$, Mann-Whitney rank sum test). Nonparametric statistical testing was used throughout these experiments because of unequal variance among several data groups of interest.²³ The effect of including 100-fold molar excess cold BDNF in the collicular injections was to decrease the observed grain density in control contralateral eyes (normal IOP) by 28% (Table 1; $P = 0.013$, Mann-Whitney rank sum test).

We calculated the difference between grain density with and without cold BDNF competition for the major outcome groups. The nonradiolabeled BDNF would be expected to compete with radiolabeled BDNF for specific receptors on RGC axonal membranes in the colliculus. The decrease caused by this competition would be an estimate of specifically transported BDNF and its alteration by elevated IOP. When we subtracted the grain density with cold competition from that in the hot BDNF group, the contralateral eyes with elevated IOP (PP25) had 74% lower density compared with normal IOP eyes (Table 1).

Among eyes ipsilateral to the collicular injection that had normal IOP, there was a substantial reduction in grain density when nonradiolabeled BDNF was added to the collicular injection (67% less than the hot BDNF group, $P = 0.06$; Table 2). When ipsilateral eyes with IOP equal to blood pressure were compared with those with normal IOP, the total grain density was reduced, even more than at the lower IOP elevation in contralateral eyes. The data for ipsilateral eyes were calculated to estimate the decrease in specifically transported BDNF, as described for contralateral eyes, by subtracting the grain density with cold BDNF competition from the control retinal density with radioactive BDNF injection alone in both elevated IOP and normal IOP eyes. The percentage of reduction from control at PP0 was 83.0% (Table 2).

If axonal transport were obstructed with IOP increase, the location of the blockage would be expected to be near the optic nerve head. In monkey and human eyes, the blockage was within the nerve head at the lamina cribrosa.⁴ In rat eyes contralateral to collicular injection, there was a definite difference in optic nerve head and nerve grain density between elevated and normal IOP groups. This consisted of a decrease from control grain density in the optic nerve head and unmyelinated optic nerve adjacent to it and an increase in grains overlying the myelinated optic nerve farther behind the eye (Table 3). For optic nerve data, we subtracted the background estimate for each eye taken from two areas overlying photoreceptors in the retina. Median grain densities were substantially decreased in the nerve head and the unmyelinated portion of the nerve that adjoins it. The overall grain densities were depressed there by 31.3% and 86.8% compared with control, whereas their respective change in specifically transported BDNF (as calculated above for retinal grains) were reduced by 98%. The decrease at the optic nerve head was statistically significant ($P = 0.05$, Mann-Whitney rank sum test), but that in the unmyelinated nerve did not reach significance at 0.05. By contrast, the myelinated optic nerve showed a median increase in grain density with IOP increase. Compared with controls with normal IOP, there was a 350% mean increase in grain density and a 671% increase in BDNF estimated to be specifically transported and present at this site after IOP elevation. Because of low grain densities in this area, the increase in

FIGURE 2. Optic nerve head of normal rat eye 6 hours after intracollicular injection of radiolabeled BDNF. This eye was contralateral to injection and was at normal IOP. (A) In a low-power view, grains appeared as tiny white dots that were numerous over the nerve fiber layer of the retina (seen at higher power in B), were fewer over the midoptic nerve head (higher power in C), and were still fewer over the unmyelinated optic nerve segment at the bottom (higher power in D). Larger clear oval areas are axons. Phase contrast; bar, (A) 100 μm ; (B) through (D), 50 μm .



myelinated nerve counts did not achieve statistical significance with the number of rats that were studied.

We suspected from prior experiments that some of the radioactivity detected at the retina had arrived by diffusion from the cerebrospinal fluid or through the blood stream. The latter could occur despite a known failure of BDNF to penetrate the blood-brain barrier¹³ if the normal blood-brain or blood-retinal barriers were not intact in some of our experimental procedures. Certainly, the fact that considerable NFL radioactivity was still present in the cold competition group supports the possibility of nonaxonal transport mechanisms. As seen in Table 2, there were still grains in eyes in which IOP was high enough to stop blood flow (a condition in which axonal transport must be blocked) or in which the optic nerve had been transected. However, in eyes in which the optic nerve was not only transected but also ligated with suture, the retinal grain density was 33% of that in eyes with transection alone (median for 6 eyes, 216 grains/10,000 μm^2 compared with 663 grains/10,000 μm^2 for 12 eyes with transection alone; $P = 0.2$, Mann-Whitney rank sum test). It is relevant to this finding that in many specimens, there were abundant grains overlying the meninges surrounding the optic nerves.

DISCUSSION

We found a reduction in axonally transported BDNF to the retinal NFL at IOP 25 mm Hg below the systolic blood pressure. This level of IOP did not cause acute alterations in the light microscopic appearance of the retina, suggesting that gross ischemia was not the cause of the transport interruption

in these eyes. Whether poor vascular nutrition, mechanical injury, or a combination of factors was operative as the cause of transport blockade is beyond the scope of this study. There was severe retinal swelling seen in ipsilateral eyes with IOP set equal to systolic blood pressure, indicating that acute ischemia was caused at these extreme IOP levels. The decrease in radiolabeled BDNF in eyes with very high IOP (PP0) compared with controls demonstrated that transport occurs and that it can be interrupted with severe conditions. The fact that transport was interrupted in the contralateral eyes with IOP set 25 mm Hg below blood pressure, at an average IOP of 50 mm Hg, is potentially more relevant to chronic human glaucoma. These experiments were designed to demonstrate whether acute experimental IOP causes a block of BDNF transport. Future

TABLE 1. BDNF Grain Density in Retinal Contralateral to Collicular Injection

	All Eyes	Hot Only	Hot Minus Cold Competition	Reduction by Competition
Control (n)	1021 (18)	1059 (9)	291 (9)	
PP25 (n)	637 (18)	843 (8)	75 (8)	
Block (%)	-38*		-74*	-28†

Data are median values for retinal data in grains/10,000 μm^2 . PP, perfusion pressure equal to difference between systolic blood pressure and eye pressure; hot only, animals receiving only radiolabeled BDNF; reduction by competition is the proportionate decrease in grain density in cold competition controls compared with hot-only controls.

* $P < 0.001$; † $P = 0.013$; both Mann-Whitney rank sum test.

TABLE 2. BDNF Grain Density in Retina Ipsilateral to Collicular Injection

	Cold Competition	Hot Only	Reduction by Surgery (%)	Reduction by Competition (%)	P
Control (<i>n</i>)	387 (6)	1181 (5)		-67.2	0.05
PP0 (<i>n</i>)	474 (4)	522 (4)	-83.0		0.06
Transection (<i>n</i>)	216 (10)	546 (8)	-80.0		0.59

Data are median retinal grain density for each group in grains/10,000 μm^2 . *P* is probability derived from the Mann-Whitney rank sum test. Reduction by competition is the proportionate decrease in grain density in cold competition controls compared with hot-only controls. Reduction by surgery is the effect of PP0 (eye pressure equal to systolic blood pressure) or transection on specific BDNF transport, calculated by (surgery group - cold competition control)/(hot-only control - cold competition control).

studies will examine whether chronic lower IOP elevations are also associated with such transport disruption.

Our previous investigations had suggested that BDNF transport from sites outside the eye into the retina existed and was altered by experimental glaucoma. In both monkey and rat eyes with elevated IOP, we found accumulation of the TrkB receptor protein for BDNF at or behind the optic nerve head in areas that corresponded to zones of vesicle accumulation by electron microscopy. It is known that TrkB receptors are transported retrograde in membrane-bound vesicles in a variety of neuronal systems.^{7,17} The present experimental results document conclusively that retrograde transport of BDNF occurs from the primary central target area for RGCs in the adult rat, the superior colliculus, and that it is interrupted by moderate IOP elevation. Because BDNF is an important trophic factor in RGC survival in development and in disease states, it is logical that IOP elevation could act to decrease RGC health if central sources of BDNF are important in the adult eye. It is known that RGCs produce their own BDNF, and there are probably other sources of supply for this and other neurotrophins.^{24,25} However, it is not known whether endogenous BDNF has the same or different effects on RGCs as does BDNF that is retrogradely transported from target cells in a complex with its TrkB receptor. We propose that failure of BDNF supply to RGCs could be a contributing feature of glaucomatous damage. Further contributing evidence for this hypothesis could be supplied if it were determined that RGCs die after experimental deprivation of retrograde BDNF transport by a mechanism other than IOP elevation.

There has been active interest in rodent models of glaucoma in the past 5 years,¹⁸⁻²⁰ because of the possibility that new approaches for RGC protection will aid in visual preservation in human glaucoma. To test such neuroprotective methods, we need relatively inexpensive animal models. Chronic IOP elevation in rats has been achieved with at least three techniques, and RGC death has been documented in these models. The rat retina and optic nerve have similarities and differences from those of monkey and human eyes. The sclera is much thinner in rats, and the optic nerve contains only 10% as many fibers. Furthermore, the rat optic nerve head has much less connective tissue in the area corresponding to the lamina cribrosa than does the monkey or the human eye.²⁶ This could be relevant to where and how axons are injured, because the regional structure of the lamina cribrosa appears to provide an explanation for the selective loss of upper and lower optic nerve fibers in glaucoma.²⁷

In the primate, the myelination of the optic nerve begins just behind the lamina cribrosa. In rats, the optic nerve is unmyelinated for the first 1 to 2 mm behind the sclera. In detailed electron microscopic studies of experimental IOP elevation in rat eyes, we found that ultrastructural features compatible with axonal transport obstruction were present in both the unmyelinated and the more central myelinated optic nerve areas.²¹ These included dilation of axons and accumulation of membrane-bound vesicles. In monkeys, similar features are confined to the lamina cribrosa of the nerve head and are not seen in the myelinated optic nerve.²⁸

TABLE 3. Optic Nerve Grain Density in Eyes Contralateral to Collicular Injection

	Cold Competition	Hot Only	Transport Change (%)	Reduction by Competition (%)	P
Nerve head					
Control	8.5	225.8		-96	0.06
PP25	14.0	12.5	-98		0.05
Unmyelinated optic nerve					
Control	16.2	59.5			
PP25	2.5	8.0	-119		0.25
Myelinated optic nerve					
Control	1.0	1.7			
PP25	3.5	6.4	+671		0.5

Data are median retinal grain density for each group in grains/10,000 μm^2 . *P* is the probability for difference derived from the Mann-Whitney rank sum test of median values. *N* = 18 rats in each control group and 17 rats in each group with PP25. Transport change is the effect of PP25 (IOP 25 mm Hg below systolic blood pressure) on specific BDNF transport, calculated from (PP25 group - cold competition control)/(hot-only control - cold competition control). Reduction by competition is the proportionate decrease in grain density in cold competition controls compared with hot-only controls.

The anatomic difference in experimental rat and primate glaucoma was mirrored in our retrograde transport data. The data from the optic nerve showed that high levels of radioactive BDNF accumulated in the myelinated optic nerve, but the levels in the nerve head and unmyelinated segment were below control density. This suggests that the blockade in the rat eye is nearer to the myelin line (2 mm behind the eye). In monkeys with acute experimental glaucoma, retrograde transport of horseradish peroxidase was interrupted within the lamina cribrosa.³ In our opinion, this difference does not invalidate the use of rats for studies of the effects of experimentally elevated IOP. However, the interspecies differences make it likely that some aspects of glaucomatous damage differ in rats and primates.

We found that radiolabeled BDNF that was presented unilaterally to the rat superior colliculus arrived at the retina by both specific axonal transport and nonaxonal transport avenues. The specificity of transport was shown by the decrease in retinal and optic nerve grain density with competition for available receptor transport by an excess of nonlabeled BDNF. In previous experiments, we found that this competitive inhibition was specific for BDNF and was not present with excess unlabeled nerve growth factor coinjection. In addition, there were high levels of radioactivity in the NFL and surrounding RGCs and almost none in the remainder of the retina. This speaks against blood-borne movement of BDNF or simple diffusion from outside the eye. Yet, we found measurable radioactivity even in eyes with transected optic nerves. When this was observed in a previous experiment, we suspected that high levels of radioactive BDNF could be present in the ipsilateral optic nerve from direct axonal transport, from vascular transport, or from diffusion through the cerebrospinal fluid from the injection site. After transection alone, material might hypothetically pass across the transection gap, attaining the retina by further diffusion or by continued transport in axons on the side of the transection attached to the eye. This speculation appears to be confirmed by the dramatic decrease in nontransported BDNF caused by suture ligation of the transected nerve stump.

The pattern of high levels of BDNF in the retinal NFL compared with the optic nerve suggests that transport brings the neurotrophin rapidly to the eye with little attrition or local slowdown along the nerve. Once in the retina, high levels of BDNF were present in the NFL and the RGC layer. It is not possible with the methods used here to determine whether the BDNF that arrived at the RGC layer remained within RGC bodies alone. It is possible that some label may have been present within other retinal neurons or glia.²⁹ Recent research suggests that alterations in BDNF may be important in experimental glaucoma³⁰ and that other neurotrophins may be important to survival of RGCs under stress. These include ciliary body-derived neurotrophic factor (CNTF).³¹

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