Elevated mRNA Expression of Brain-Derived Neurotrophic Factor in Retinal Ganglion Cell Layer After Optic Nerve Injury

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Purpose. Recent studies show that exogenous brain-derived neurotrophic factor (BDNF) can promote retinal ganglion cell survival in vivo and in vitro. BDNF is expressed by a subpopulation of cells in the ganglion cell layer (GCL). To investigate whether endogenous BDNF may play a role in neuronal protection after ganglion cell trauma, BDNF expression in the retina was examined after optic nerve (ON) injury.

Methods. The optic nerve in Sprague-Dawley rats was crushed intraorbitally posterior to the optic disc. For controls, the optic nerve on the opposite side in each animal was similarly exposed but was not crushed. After intervals of 6 hours to 6 weeks, eye tissues were processed for in situ hybridization, Northern blot, and RNase protection assay using radiolabeled rat riboprobes.

Results. After ON injury, BDNF expression was significantly elevated in cells restricted to the GCL, and more cells demonstrated expression of BDNF than were observed in the controls. Elevated BDNF expression was first observed at 24 hours, peaked at 48 hours, and declined to the basal level 2 weeks after ON injury. Quantitative analysis showed a fivefold to sixfold increase in the number of BDNF-positive cells and a 54% increase in BDNF signal intensity in individual cells in the GCL 48 hours after ON injury. In control retinas without ON injury, BDNF expression was localized to some cells in the GCL, as was observed in normal eyes without surgery. Northern blot and RNase protection assay demonstrated a 38% elevation in BDNF expression above control levels 48 hours after ON injury.

Conclusions. These results indicate that cells in the GCL can upregulate gene expression of BDNF in response to ganglion cell axonal injury and suggest that endogenous BDNF may contribute to a natural neuroprotective process after ON injury. Invest Ophthalmol Vis Sci. 1997;38:1840–1847.

Neurotrophic factors are key elements in the regulation of neuronal development and are required for maintenance in the adult nervous system. Among them is the family of the neurotrophins that includes nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophins 3, 4/5, and 6. BDNF is the most abundant neurotrophin in the adult brain.1,2 Many studies have shown that exogenous BDNF can promote survival and prevent neuronal death of retinal ganglion cells (RGCs) after axotomy in the optic nerve3–6 or in cell culture.7–9 Exogenous BDNF can enhance optic axon branching and remodeling in vivo10 and can protect ganglion cells from retinal ischemic injury.11

Although these studies suggest that exogenous BDNF plays important roles in RGC integrity, little is known about the function or regulation of endogenous BDNF expression in the retina. Our previous study12 and others13 showed that a population of RGCs express BDNF. In the current study, we examined BDNF mRNA expression in the retina after optic nerve (ON) injury. By identifying changes in BDNF expression in the retina after ON injury, insights into a func-

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tional role of endogenous BDNF in ganglion cell maintenance and protection may be forthcoming.

Our previous studies showed that basic fibroblast growth factor (bFGF), as a retinal endogenous growth factor in the photoreceptors, is significantly upregulated by the photoreceptors when they are stressed by either inherited disorders or environmental insults such as intense or constant light. Another study showed an increase of bFGF expression after mechanical injury to the retina. Because administration of exogenous bFGF into eyes could delay photoreceptor degeneration in some mutant (Royal College of Surgeons) or light-damaged rats, it is suggested that endogenous BFGF may function as a natural photoreceptor protection or rescue factor that is activated in response to photoreceptor stress.

An obvious question arises as to whether other retinal neurons could also increase the expression of their endogenous neurotrophic factor when they encounter insults—in other words, whether upregulation of neurotrophic factor in neurons is not only a feature of the photoreceptors in the outer retina, but also a characteristic of neurons in the inner retina. To determine whether ganglion cells also respond to cellular insults by increasing their BDNF expression in a manner similar to the photoreceptors' upregulation of bFGF in response to stress, BDNF mRNA expression was examined in the retina after optic nerve injury.

MATERIALS AND METHODS

Animals

Sprague–Dawley albino rats were obtained from Charles River Laboratories (Wilmington, MA). Animals were fed ab libitum with Purina lab chow and water with room lighting consisting of a 12-hour light/12-hour dark cycle. All animals were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optic Nerve Crush Surgery

Rats 6 to 7 weeks old were anesthetized with intraperitoneal injections of pentobarbital (Nembutal, 75 mg/kg). Lateral canthotomy was performed and temporal conjunctiva and Tenon’s capsule were opened along the limbus to allow access to the abductus muscle. The abductus was then severed so that the eye could be pulled nasally to expose the posterior pole of the eye. The optic nerve was approached along the temporal scleral surface. Dull dissection was used and close attention was paid to avoid damaging blood vessels around the posterior pole of the eye. When it was exposed, the optic nerve was crushed 2 to 3 mm posterior to the optic disc using a microclip (Baby Dieffenbach Serrefines, RS-5471; Roboz Surgical Instrument, Rockville, MD). The crush was applied for 30 seconds between the tips of the microclip. After a 60-second interval, the optic nerve was crushed again at the same site for another 30 seconds, as described previously. The eye was not pulled nasally when the crush was applied. The opposite eye of each animal was used as surgery control: an identical operation was performed on the other eye, except that the optic nerve was only exposed, not crushed.

After surgery, animals were killed at 6 hours, 24 hours, 48 hours, 72 hours, 1 week, 2 weeks, 4 weeks, and 6 weeks. Two animals were used for each postinjury stage.

Tissue Preparation

Animals were killed with an overdose of pentobarbital (Nembutal). Eyes were enucleated, an incision was made in the cornea, and eyes were fixed immediately in 4% formaldehyde in 0.1 M phosphate buffer (pH = 7.4). After 15 minutes in the fixative, the lenses were removed and eyes were cut along the cornea–optic nerve axis into two halves. Tissues were further fixed and cryoprotected overnight in 4% formaldehyde, 0.5% glutaraldehyde, and 20% sucrose in 0.1 M phosphate buffer (pH = 7.4). Tissues were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and cryosectioned at a thickness of 10 μm at −21°C. Tissue sections were cut from areas within 1 mm of the optic nerve. Thus, each section contained the entire central and peripheral retina in one meridian. The tissue sections from the ON-injured and control eyes were mounted on the same slide and processed identically so that sections could be directly compared with as little processing variability as possible.

In Situ Hybridization

A rat BDNF cDNA clone was obtained as a generous gift from Genentech (San Francisco, CA). It consists of 460 bases of coding region and was inserted into plasmid pGEM-4Z.21 For generation of antisense and sense BDNF riboprobes, the plasmid was linearized with restriction enzyme Hind III, respectively. 35S-labeled antisense and sense riboprobes were transcribed using the Riboprobe Gemini System according to the manufacturer’s instructions (Promega, Madison, WI). The tissue sections were pretreated with 10 μg/ml proteinase K at 37°C for 20 minutes and 0.25% acetic anhydride and 0.1 M triethanolamide for 10 minutes. The tissue sections were then incubated at 50°C on a slide warmer for 18 ± 2 hours with the probe solutions containing 5 × 10⁶ cpm/ml 35S-labeled probes, 50% formamide, 10% dextran sulfate, 500 mM NaCl, 0.5 mg/ml tRNA, 10 μM dithiothreitol, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bo-
Three or four sections were counted for each eye, with a light microscope through an Hitachi video camera and two animals were used for each postinjury stage. The number of BDNF-positive cells in the retinas was determined in the GCL for each section. More BDNF-positive cells were present in the GCL compared to the control retina without ON injury (I). However, the number of BDNF-positive cells and the silver grain density over individual cells were lower at 72 hours than at 48 hours after injury (D, E). No significant difference was detected in silver grain density between ON-injured (F) and control retinas (I). Significantly increased silver grain density was observed over individual BDNF-positive cells in the GCL. More BDNF-positive cells were present in the central retina (left) than in the more peripheral retina (right). Seventy-two hours after ON injury (E). More BDNF-expressing cells were present in the GCL compared to the control retina without ON injury (I). However, the number of BDNF-positive cells and the silver grain density over individual cells were lower at 72 hours than at 48 hours after ON injury (D). One week after ON injury (F). There were still more BDNF-positive cells than in the control retina (I), but the silver grain density over individual cells was much lower at 48 or 72 hours after injury (D, E). No significant difference was detected in silver grain density between ON-injured (F) and control retinas (I). Two weeks after ON injury (G). Only a few cells were positive for BDNF, as observed in the control retinas without ON injury (I). Four weeks after ON injury (H). No BDNF signal was detected in the retina. However, in the control retina (I), BDNF signals were present in a small population of cells, as observed in other controls. BDNF signals in control retinas without ON injury. A small population of cells in the GCL was positive for BDNF, similar to those observed in normal retinas (A). Retinas with or without ON injury labeled with sense rat BDNF riboprobe. No signal was detected in the retinas. All micrographs are at identical magnifications. Scale bar = 200 μm.

**FIGURE 1.** Brain-derived neurotrophic factor (BDNF) mRNA expression in the rat retina after optic nerve (ON) injury using in situ hybridization. (A) In normal retina without any surgery, BDNF hybridization signals were present in a subpopulation of cells in the ganglion cell layer (GCL; arrowheads). (B) Six hours after ON injury. BDNF signals were observed in a small population of cells in the GCL, and no significant difference was detected when compared to the normal retina. (C) Twenty-four hours after ON injury. More BDNF-expressing cells were observed in the GCL compared to the control retina without ON injury (I). (D) Forty-eight hours after ON injury. Maximal number of BDNF-expressing cells was observed at this time after ON injury. Significantly increased silver grain density was observed over individual BDNF-positive cells in the GCL. More BDNF-positive cells were present in the central retina (left) than in the more peripheral retina (right). (E) Seventy-two hours after ON injury. More BDNF-expressing cells were present in the GCL compared to the control retina without ON injury (I). However, the number of BDNF-positive cells and the silver grain density over individual cells were lower at 72 hours than at 48 hours after ON injury (D). (F) One week after ON injury. There were still more BDNF-positive cells than in the control (I), but the silver grain density over individual cells was much lower than at 48 or 72 hours after injury (D, E). No significant difference was detected in silver grain density between ON-injured (F) and control retinas (I). (G) Two weeks after ON injury. Only a few cells were positive for BDNF, as observed in the control retinas without ON injury (I). (H) Four weeks after ON injury, no BDNF signal was detected in the retina. However, in the control retina (I), BDNF signals were present in a small population of cells, as observed in other controls. (I) BDNF signals in control retinas without ON injury. A small population of cells in the GCL was positive for BDNF, similar to those observed in normal retinas (A). (J) Retinas with or without ON injury labeled with sense rat BDNF riboprobe. No signal was detected in the retinas. All micrographs are at identical magnifications. Scale bar = 200 μm.
membranes (Schleicher & Schuell, Keene, NH) and hybridized to \(^{32}\)P-labeled BDNF probe \((3 \times 10^6 \text{ cpm/ml})\). The membrane then was washed in graded SSC, dried, and exposed to PhosphorImager plate (Molecular Dynamics, Eugene, OR). Relative abundance of mRNA was quantified by reading the plate. For accurate quantification, the same blot was stripped off and hybridized to \(^{32}\)P-labeled \(\beta\)-actin probe. The ratio of BDNF to \(\beta\)-actin densities then was used for comparison between ON-injured and control groups.

Because the abundance of BDNF mRNA in the retinas was very low, RNase protection assay, a more sensitive technique to detect the signal, was used. RNase protection assay was performed using the RPA II system (Ambion, Austin, TX) according to the manufacturer’s procedure. Briefly, total RNA of 30 \(\mu\)g isolated from retinas 48 hours after surgery (with or without ON injury) and from normal retinas was hybridized with \(^{32}\)P-labeled antisense rat BDNF cRNA probes \((5 \times 10^6 \text{ cpm})\) at 43°C for 18 ± 2 hours. Unhybridized probe was then degraded with 2.5 U/ml of RNase A and 100 U/ml of RNase T1 for 30 minutes at 37°C. The samples were then separated on 5% acrylamide/8 M urea gel and visualized by autoradiography on x-ray film. Full-length major protected bands were quantitated using the PhosphorImager plate (Molecular Dynamics). \(\beta\)-actin mRNA expression was also examined and used as an internal control.

**RESULTS**

In the normal adult rat retina, BDNF mRNA expression was present in a subpopulation of cells in the GCL (Fig. 1A). When quantitated, BDNF-positive cells
accounted for 5% to 6% of the cells in the GCL (Fig. 2) and were randomly distributed throughout the retina (Fig. 1A). Six hours after ON injury, no significant change of BDNF expression was observed in the retina (Fig. 1B). The number of BDNF-positive cells and signal intensities in individual cells were very similar between ON-injured and control groups (Fig. 1I).

Twenty-four hours after ON injury, BDNF expression was elevated (Fig. 1C). The number of BDNF-positive cells in the GCL increased to 10%, whereas in the control group only 5% to 6% of the cells were labeled (Fig. 2). The BDNF signal intensity was also significantly higher in individual cells in the ON-injured group, as evidenced by increased density of silver grains (Fig. 1C).

BDNF expression peaked 48 hours after ON injury (Fig. 1D). The number of BDNF-positive cells in the GCL increased to 28% (24% to 31%) compared to the control group, in which only 5% to 6% were labeled with BDNF probe (Fig. 2). More BDNF-positive cells were present in the central retina than in the peripheral retina (Fig. 1D). To confirm that elevated BDNF expression was caused not only by the presence of more BDNF-expressing cells after ON injury but also by increased expression in individual cells, BDNF signal intensities in individual cells were quantitated (see Materials and Methods), and ON-injured and control groups were compared. Quantitative analysis showed a 54% increase in silver grain density over individual cells in the ON-injured group than in the control group at 48 hours after ON injury (Fig. 3). These results indicate that BDNF expression levels increase in individual GCL cells after ON injury.

BDNF expression in ON-injured retinas declined from peak levels at 48 hours. Seventy-two hours after ON injury, BDNF expression was lower than at 48 hours but still significantly elevated (Fig. 1E) when compared to the control retinas (Fig. 1I). The number of BDNF-positive cells in the GCL was 23%; in the control retinas, only 5% to 6% of cells in the GCL were positive for BDNF (Fig. 2), comparable with that in normal retinas. The signal intensity in individual...
cells at this stage was lower than at 48 hours but still significantly higher than in control retinas (Fig. 1E). BDNF-positive cells were more abundant in the central than in the peripheral retinas, as observed at 48-hour stage.

One week after ON injury, the number of BDNF-positive cells and signal intensity in individual cells continued to decline (Fig. 1F), but there were still more cells positive for BDNF than in the control group. When quantitated, 12% of cells in the GCL were positive for BDNF in the ON-injured group; in the control group, only 7% of cells were labeled (Fig. 2). However, the signal intensity in individual cells was much lower at this time than at 48 or 72 hours after ON injury (Figs. 1D to IF). No significant difference was observed in the signal intensity in individual cells between ON-injured and control retinas (Figs. 1F, IF).

Two weeks after ON injury, BDNF expression returned to basal level (Fig. 1G). Fewer than 4% (3.4%) of cells in the GCL were positive for BDNF, comparable to the 3.3% figure in the control group (Fig. 2). The signal intensities in individual cells were also comparable between the two groups (Figs. 1G, IG).

BDNF expression in the retinas continued to decline after 2 weeks after ON injury. Four weeks after ON injury, BDNF expression declined to a level where no obvious signal could be detected in the retinas using the in situ hybridization technique (Fig. 1H). However, in the control retinas, BDNF expression was still present in a small population of cells, as observed in other control stages (Fig. 1I). Similar findings were noticed at the 6-week stage, in which no BDNF signal was observed in ON-injured retinas (data not shown) but the signal was detected in the control retinas and was comparable to other control stages.

Analysis of variance (nested effects model) was used to determine the difference between ON-injured and control groups in the number of BDNF-positive cells in the GCL. No significant difference was found at 6 hours and 2 weeks after ON injury. There were significantly more BDNF-positive cells in the ON-injured group than in the control group at 24 hours, 48 hours, 72 hours, 1 week, and 4 weeks, with $P < 0.025$, 0.001, 0.001, 0.01, and 0.025, respectively.

To confirm the in situ hybridization results, Northern blot and RNase protection assay were performed. The stage of 48 hours, in which retinas showed peak levels of BDNF mRNA expression after ON injury in in situ hybridization, was chosen to confirm the upregulation of this neurotrophic factor. RNAs were isolated from retinas with or without ON injury and used in the assay. RNAs from normal adult retinas without any surgery were also used as a control. Analysis of variance showed a significant 38% increase of BDNF mRNA expression in the retinas with ON injury than in the control retinas without ON injury; BDNF expression levels were comparable in normal retinas and in the control retinas without ON injury (Fig. 4). $\beta$-actin mRNA expression was also examined and used as an internal control. No difference was detected in actin expression among the three groups. These results are consistent with the in situ hybridization study results and show that BDNF mRNA expression levels were significantly elevated in the retinas after ON injury.

**DISCUSSION**

The results of this study clearly demonstrate that BDNF mRNA expression undergoes significant elevation in a subpopulation of cells in the GCL after ON crush injury. The upregulation of BDNF expression was first detected at 24 hours (Fig. 1C), peaked at 48 hours (Fig. 1D), lasted about 1 week (Fig. 1F), and returned to basal level 2 weeks after ON injury (Fig. 1G). Increased BDNF expression was confirmed by Northern blot and RNase protection (Fig. 4). This increase was the result of more BDNF-expressing cells and increased signal intensity in individual cells after ON injury (Fig. 4).

Because it has been reported that as many as 40% to 50% of cells in the GCL are displaced amacrine cells in the adult rat retina, an obvious question arises as to the type of cells involved with BDNF expression in the GCL. If some BDNF-positive cells
in the GCL were amacrine cells, it is reasonable to expect that some amacrine cells in the inner nuclear layer should also be labeled with BDNF probe, but this was not observed: No BDNF hybridization signal was detected in the inner nuclear layer. To our knowledge, there has been no report that ON injury can cause changes in amacrine cells, whereas ganglion cell changes after ON injury have been well documented.

Because ON crush may cause occlusion of the central retinal artery, it is possible that transient ischemia may contribute to the change in BDNF expression observed in the GCL. This study cannot exclude this possibility. However, previous studies do not support the possibility that ischemia plays a major role in this BDNF upregulation. Other studies showed that a 60-minute period of complete occlusion of the central retinal artery is required to demonstrate mild histologic ischemic damage in the rat retina; a 30-minute occlusion did not result in any histologic change. 23-25 Our study used two 30-second episodes of ON crush intercalated by a 60-second noncrush period, not long enough to produce significant ischemic damage to the GCL when compared to those other studies. 20 Another analysis showed that bilateral common carotid artery occlusion for 2 minutes resulted in increased BDNF expression in dentate gyrus granule cells but not in any other area in the brain. 26 Therefore, it seems unlikely that the increased BDNF expression in our study was due to an ischemic effect.

To determine whether BDNF upregulation is a unique phenomenon in the retina after ON injury, we also examined nerve growth factor, neurotrophin 3, and trkB receptor mRNA expressions using in situ hybridization. No significant change was detected in the expression of these growth factors or receptor after ON injury (data not shown). Thus, BDNF is the only neurotrophin examined that demonstrated increased mRNA expression in this study.

What is the significance of elevated BDNF expression after ON injury? A recent study showed that after intraorbital ON transection in the adult rat, all RGCs survive for 5 days, but 90% of them die by 2 weeks. 27 The time course of RGC survival period after ON transection correlates well with the BDNF upregulation time in our study. Because exogenous BDNF could promote RGC survival and delay RGC death in vivo 28-29 and in vitro, 30-32 it is reasonable to speculate that elevated expression of endogenous BDNF may play a similar role. Increased BDNF expression may reflect the initiation of a natural protective pathway soon after ON injury. Endogenous BDNF may eventually fail to protect RGCs because axon damage is too severe to be altered by the protective effects of increased BDNF production, or because BDNF produced in the GCL does not provide sufficient protection, because exogenous BDNF could delay RGC death. 4-6

In summary, this study provides the first evidence that cells in the GCL respond to ON injury by upregulating BDNF expression. These results suggest that endogenous BDNF may act as a natural survival or protection factor in the ganglion cells when they encounter injury. Because the expression of bFGF, an endogenous growth factor in the photoreceptors, is upregulated when the photoreceptors encounter either inherited or environmental insults, 14-16 we propose that upregulation of endogenous neurotrophic and neuroprotective factors is a general phenomenon in both the outer and inner retinas during neuronal degeneration.

**Key Words**

brain-derived neurotrophic factor, neurotrophins, optic nerve injury, retina, retinal ganglion cells

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