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BRAIN_DERIVED NEUROTROPHIC FACTOR PROMOTES RETINALGANGLION CELL SURVIVAL AND REDUCES TYROSINE RECEPTOR KINASE B PROTEIN AND MRNA IN VIVO

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By

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ABSTRACT

BRAIN-DERIVED NEUROTROPHIC FACTOR PROMOTES RETINAL GANGLION CELL SURVIVAL AND REDUCES TRYROSINE RECEPTOR KINASE B PROTEIN AND mRNA IN VIVO

By

Hao Chen

Glaucoma is a disease of the visual system that often is characterized by elevated intraocular pressure, compressive damage to optic nerve (ON), and a loss of ganglion cells within the retina. Since direct mechanical damage to the ON also results in a progressive loss of ganglion cells, ON crush is commonly used as a model to evaluate treatment strategies for different retinal disorders. Brain-derived neurotrophic factor (BDNF) has been shown to be one of the most effective neuroprotectants in the rat eye, a fact confirmed by the present studies (94% survival rate in treated eyes).

Since the rodent and primate eyes differ in many respects, a cat optic nerve crush model was developed as an intermediate test of whether BDNF might also serve as an effective neuroprotectant in primate-sized eyes. Here, treatment with 15, 30, 60, and 90μg of BDNF at the time of the ON crush resulted in ganglion cell survival rates of 52%, 81%, 77%, and 70%, respectively. Cell size comparisons showed that 30μg saved a wider range of ganglion cells, while 90μg minimized the loss of medium-sized neurons and retained normal proportions of large, medium, and small ganglion cells.

To test whether Müller cells, the primary glia of the retina, are affected by ON injury or are involved in BDNF-mediated responses, two glia specific proteins were

studied. Glia fibrillary acidic protein (GFAP) increased ~7-9 fold 3 days post ON crush and remained elevated for the 2 week period examined. Glutamine synthetase (GS), an enzyme typically located within Müller cell bodies, showed a temporary shift from the cell soma to the Müller endfeet of the inner retina within 24 hours. The change was not accompanied by an increase in total GS protein. BDNF, however, did not affect GFAP or GS levels, suggesting that Müller cells do not contain BDNF receptors.

The biological effects of BDNF are mediated primarily by its high affinity receptor tyrosine kinase B. Although this receptor is present in both truncated and full length form, it is the full length receptor (TrkB-FL) that is responsible for initiation of the intracellular signaling cascade that underlies the neuroprotective role of BDNF. Interestingly, intravitreal application of BDNF results in a rapid, dramatic, and long lasting reduction in TrkB-FL protein in the normal rat retina; only 4.8% and 31.4% of protein remains at 1 and 14 days post-injection, respectively. Optic nerve crush reduced TrkB-FL protein 14 days post-crush. When BDNF is applied at the time of the ON nerve crush, TrkB-FL protein decreases to levels similar to that seen in animals receiving the BDNF injection alone. These data suggest that the failure to enhance RGC survival in the cat via multiple injections of BDNF most likely resulted from a BDNF-induced reduction in the overall level of full length receptors within those eyes. In all cases, the changes in TrkB-FL protein were accompanied by changes in TrkB-FL mRNA levels as well. The pattern of change in TrkB-FL mRNA followed a bell-shaped curve, with that measured on days 1 and 14 below normal.

In summary, the results of these experiments provide encouraging data indicating that BDNF is a potential neuroprotectant for use in glaucoma patients.

Dedicated to my parents and my wife

For their endless love and support

And to Ms. Dorothy Schmidt

For giving me a home away from home

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LIST OF ABBREVIATIONS

BDNF	-- Brain-derived neurotrophic factor
CREB	-- cAMP response element-binding protein
ERK	-- extracellular signal-regulated kinase
GFAP	-- Glia fibrillary acidic protein
GS	-- Glutamate synthetase
INL	-- Inner nuclear layer of the retina
L-NAME	-- N-ω-nitro-L-arginine-methylester
NGF	-- Nerve growth factor
NMDA	-- N-methyl-D-aspartate
NO	-- Nitric oxide
NOS	-- Nitric oxide synthase
NT-3	-- Neurotrophin –3
NT-4	-- Neurotrophin –4
ON	-- Optic Nerve
PI-3K	-- phosphatidyl-inositol-3'-kinase
RGC	-- Retinal ganglion cell
RT-PCR	-- Reverse transcription polymerase chain reaction
SDS-PAGE	-- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
trkB-FL	-- Full length trkB mRNA
TrkB-FL	-- Full length TrkB protein

Chapter I

Brain-Derived Neurotrophic Factor Promotes the Survival of Retinal Ganglion Cells after Optic Nerve Injury in Adult Rats

INTRODUCTION

Glaucoma is a group of diseases often characterized by increased intraocular pressure (IOP), structural changes in the appearance of the optic disk at the back of the eye, visual field defects, and progressive blindness. Glaucoma has been recognized as a major cause of blindness for at least 1000 years. It is estimated that at present glaucoma affects ~3 million people in the United States alone, and that as many as 120,000 Americans are legally blind as a result of the disease.

The loss of vision in glaucoma is due primarily to the degeneration of one particular type of retinal neuron called the retinal ganglion cell (RGC) and its axon. The RGC is one of five types of neurons found in the retina. The others are photoreceptors (rods and cones), bipolar, horizontal, and amacrine cells. These different types of neurons divide the neural retina into five layers. The cell bodies of photoreceptors are reside in the outer nuclear layer (ONL) and are located farthest from the incoming light. Photoreceptors are the only photosensivitive elements in the retina. They convert light energy into electrical signals, which then are relayed to the bipolar cells. Bipolar cells are located in the inner nuclear layer (INL), together with other interneurons of the retina, namely horizontal and amacrine cells. Horizontal cells receive input from the photoreceptors and provide lateral inhibition and spatial contrast. Amacrine cells receive

their input primarily from bipolar cells, and influence the temporal response of RGCs. Retinal ganglion cells are located in the inner surface of the retina. The RGCs integrate visual information from bipolar and amacrine cells, and their axons exit the eye at the optic disc and form the optic nerve and tract. Post synaptic targets of the RGC axons include the lateral geniculate nucleus (LGN), the superior colliculus, the pretectum, and the hypothalamus. Interposed between the neuronal layers of the retina are the inner and outer plexiform layers (IPL, OPL, respectively), where synaptic interactions among retinal neurons occur.

In addition to neurons, the retina also contains a number of different types of glial cells. The primary glial cell of the retina is the Müller cell. Müller cells are unique to the retina, and are considered to be specialized astrocytes. Their cell bodies are located in the INL along with those of the bipolars and amacrine cells, and their processes extend across the full width of the retina. In addition to Müller cells, the retina also contains astrocytes and microglia.

The elevation of IOP commonly associated with glaucoma results from a build up of aqueous humor within the anterior segment of the eye. Aqueous humor, which is similar to plasma, is continuously produced by the epithelial layer of the ciliary body. It provides nutrients and removes metabolites for the avascular lens and cornea. Aqueous humor exits the eye through the trabecular meshwork and Schlemm's canal located in the anterior chamber angel located between the cornea and the iris. The aqueous then drains into episcleral veins. Under normal conditions, IOP is maintained at ~15mmHg by a balance in the production and outflow of aqueous (~1.5µl/min at night and 2.5µl/min during the day). In the case of glaucoma, the build up of aqueous humor appears to result

from a decrease in outflow facility, and not from over production. For example, in primary open angle glaucoma, the most common form of glaucoma (~90% of all glaucoma cases in the USA), the rise in IOP results from blockage of the drainage channels in the trabecular meshwork by cellular debris. An increase in IOP also occurs when the iris becomes displaced and blocks the trabecular meshwork, resulting in closed angel glaucoma (<10% of all cases).

Chronic elevation of IOP, combined with periods of abnormal fluctuations in pressure, is thought to initiate glaucomatous neuropathy by damaging the optic nerve as it exits the eye. Since the optic nerve comprises the axons of retinal ganglion cells, this pressure-induced axonal injury also leads to the degeneration of these neurons within the retina itself. Degeneration of RGCs has been demonstrated in human glaucoma patients (see review, ref [1,2]) as well as experimental glaucoma in primates [3], rabbits [4,5] and the rat[6,7].

In addiction to RGC degeneration, we recently demonstrated that neuronal degeneration also occurs in the lateral geniculate nucleus, the primary target area of RGC axons in primates and humans. Elevation of IOP in one eye resulted in a 38% decrease in the number of LGN neurons in the corresponding magnocellular layers of the LGN, whereas the parvocellular layers were not affected significantly. The cross-sectional areas of LGN neurons receiving input from the glaucomatous eye also were reduced between 10-58%, while the volume of the LGN decreased ~23-55%, depending on the durations and mean level of elevated IOP.

At present, there are several hypotheses concerning the mechanism by which elevated IOP induces glaucomatous neuropathy. One is that elevated IOP increases the

shearing stress within the lamina cribrosa, a sieve-like structure through which the axons of retinal ganglion cells pass as they exit the eye. The progressive remodeling of the extracellular matrix in the lamina cribrosa due to increased mechanical stress is thought to result in direct mechanical damage to RGC axons [8,9]. A second hypothesis is that RGC and optic nerve degeneration in glaucoma results from pressure-induced ischemia [10]. The optic nerve head is highly vascularized, and the small vessels within it could readily be compromised by compressive forces similar to those acting upon the nerve fibers. Astrocytes located in the optic nerve head also have been implicated as playing a role in glaucomatous neuropathy [11,12]. This suggestion is based on recent data showing that astrocytes can respond to both mechanical and ischemic stress by releasing nitric oxide, which can be neurotoxic [13].

Besides their axons, the somata of RGCs also are a potential site of initial glaucomatous damage. For example, the possibility of direct, hydrostatic pressure-induced damage on RGC somata also is under investigation. Wax et al. [14] found that cultured retinal cell lines exhibit significant alterations in their intracellular adenylyl cyclase activity following exposure to acute and sustained levels of elevated hydrostatic pressure. At present, the link between these changes and RGC degeneration remains unknown. Finally, by mounting an autoimmune response against either RGCs or their axons, the immune system too has been suggested as playing a role in RGC degeneration in glaucoma [15]. For example, antibodies against 'self' glycosaminoglycans (optic nerve head) [16], heat shock protein 60 (various retinal cells) [17], and unidentified 20kDa and 50kDa protein (ganglion cell layer) [18] have been found in some glaucoma patients.

The current strategies for alleviating glaucomatous neuropathy continue to focus on reducing IOP. The standard initial therapy typically involves the use of eye drops that either suppress aqueous production (e.g. Timolol) or increase outflow facility (e.g. Travatan and Lumigan, recently approved by the FDA). Aside from medications, surgical or laser treatment towards lowering IOP also is available. While beneficial, the effectiveness of IOP reduction in preventing additional retinal ganglion cell degeneration is not clear. Thus, several neuroprotectant-based treatment strategies are under investigation. These strategies, which have been tested mostly in conjunction with optic nerve section, include the use of free radical scavengers [19,20], glutamate receptor antagonists [21], and protease inhibitors [22]. Direct application of different neurotrophins to the injured eye also has been shown to be effective in slowing the progression of RGC death following optic nerve injury [19,23-32].

Neurotrophins are endogenous soluble proteins that regulate the survival, growth, differentiation, and protein synthesis of neurons. Members of the neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6). During the 1950s, the landmark studies on NGF by Levi-Montalcini et al. established the blueprint for subsequent studies. Neurotrophic factors stimulate a multitude of cellular events at both the structural and functional level. Such actions include stimulation of the synthesis of structural proteins, of general metabolic processes, and of neurotransmitter synthesis and release. The multiple actions of trophic factors have been characterized best in PC12 cells that, when exposed to NGF or basic fibroblast growth factor (bFGF), cease to proliferate and, over the course of several days, develop properties reminiscent of

sympathetic neurons; they extend neurites, become electrically excitable, and form synapses with muscle cells in culture.

Several members of the neurotrophin family have been tested for their ability to promote the survival of RGCs. Of these, BDNF has been shown to be one of the most effective candidate [19,25-31]. For example, Aguayo et al. [27,31] reported that a single injection of BDNF can delay the death of axotomized ganglion cells. Although RGC densities decreased in the BDNF-treated retinas at 2 weeks post axotomy, they remained significantly greater than those in untreated eyes. Similarly, Peinado-Ramon et al. [28] showed that 2 weeks after complete optic nerve transection, the mean densities of RGCs are approximately 33% higher in BDNF treated animals than the untreated controls. The neuroprotective effect seen following a BDNF injection diminishes after 5 weeks, and is lost 7 weeks after transection.

One of the long-range goals of our lab is the development of better treatment strategies for preserving RGCs in glaucoma, and following optic nerve injury in general. With this in mind, an initial goal of my thesis research was to establish an optic nerve crush model that then could be used to study RGC degeneration in the rat. Similar to the mechanical effects of elevated IOP, optic nerve crush is considered to result in ganglion cell death by disrupting the retrograde transport of trophic materials from target tissues. In addition, both optic nerve crush and experimental glaucoma have been shown to result in apoptotic, or programmed, cell death [1,7,33-37]. Based on these similarities, it is generally agreed that optic nerve crush is a reasonable model for studying the potential benefits of different neuroprotectants, such as BDNF, for use in glaucoma.

The studies presented here are divided into four Chapters. The goal of the first set of experiments was to confirm, in my hands, that BDNF is a potent neuroprotectant in the rat eye (Chapter I). I then developed a similar optic nerve crush procedure for use in the domestic cat, and derived the does-response curve necessary for achieving maximal ganglion cell survival in this primate-sized eye (Chapter II). Chapter III involved the use of immunohistochemistry and Western blot techniques to explore the role that retinal glial cells might play in RGC death following nerve injury, and finally, the regulatory effect that BDNF has on its own high affinity receptor (tyrosine receptor kinase B, TrkB), was investigated at both the protein and mRNA levels (Chapter IV).

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats weighting 250-300g were obtained from Charles River Laboratories, Inc (Wilmington, MA). Food and water were provided *ad libitum,* and the animals were maintained on a 12hr light-dark cycle. All procedures used adhered to the NIH guidelines for the use of laboratory animals, and were approved by the Animal Use Committee at Michigan State University.

The animals used in these experiments were divided into three groups: Normal (n=7), optic nerve crush only (n=3), and optic nerve crush combined with an intravitreal injection of BDNF at the time of the crush (n=3). In all animals, the retrograde tracer Fluorogold was used to identify RGCs. Two additional animals received Fluorogold

injections following the optic nerve crush in order to define the extent to which the crush injury affects axonal transport.

Retrograde Labeling of RGC by Fluorogold

Unlike RGCs in larger vertebrates, such as cats and primates, RGCs in the rat cannot be readily distinguished from other retinal neurons without pre-labeling. Thus, to ensure the accuracy in retinal ganglion cell counts, Fluorogold, a long-lasting (>20 days) retrograde neuronal tracer was used to pre-label RGCs in the normal and experimental eyes. For surgery, the rats first were anesthetized with Buprenorphine (0.4mg/kg, Reckitt & Colman Products, Richmond, VA) and pentobarbital sodium (30mg/kg, Abbott Laboratories, North Chicago, IL), and then were placed in a stereotaxic instrument. A single opening was made on each side of the skull to gain access to the superior colliculus, the primary target of RGC axons in the rat. The openings were 5mm long and 2mm wide. They were located 1mm lateral to the sagittal sinus, and were just anterior to its intersection with the transverse sinus. An incision was made in the brain beneath each opening using a 5mm long needle, and a piece of Gel-foam (Upjohn, Kalamazoo, MI) pre-soaked in Fluorogold solution (2% in sterile saline, Fluorochrome, Englewood, CO) then was inserted onto the surface of the superior colliculus. The overlying skin then was sutured, and Buprenorphine was provided as needed. Prior to receiving any further procedures, all animals received a one-week survival period to allow for retrograde labeling of the RGCs.

Surgical Procedure for Optic Nerve Crush

In preparation for the optic nerve crush, all rats were reanesthetized with Buprenorphine and pentobarbital sodium as described above. The right optic nerve was exposed by making a small incision in the lateral canthus and the nerve was crushed 1-2mm posterior to the globe for 15sec, using a pair of cross-action forceps. Care was taken to ensure that the ophthalmic artery, and therefore the retinal blood supply, was not compromised by the crush.

BDNF Treatment

Human recombinant BDNF (5μg/5μl sterile saline, Regeneron Pharmaceuticals, Inc. Tarrytown, NY) was injected into the vitreal chamber of each eye immediately following the optic nerve crush using a Hamilton syringe with a 30-gauge needle. The injections were made over a 30sec period and the needle was left in position for additional 2min to allow for diffusion of BDNF from the injection site and to minimize back flow. Sham injections were not performed, as it has been shown that injecting vehicle solution alone can promote RGC survival in rats [27]. This most likely is due to mechanical damage to structures in the anterior segment of the eye by the needle. Both the lens and ciliary body have been shown to be potential sources of endogenous neurotrophic factors [38,39]. Thus, care was taken not to damage these structures during the injection.

RGC Counts

One week following optic nerve crush, all of the rats received an overdose of Nembutal (30mg /rat) and were perfused transcardially with 150ml of saline followed by a solution containing 300ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The retinae then were dissected and flat mounted onto subbed glass slides. The tissue was defatted and dehydrated through a series of graded alcohols and xylenes, and then coverslipped with DePeX (BDH laboratory, Poole, England). RGC densities were determined by counting the number of Fluorogold-labeled neurons located at distances of 1mm, 2mm, and 3mm from the center of the optic disk in each retina quadrant, with a total of 12 areas sampled. For each sample area, one microscope field representing approximately 41,125μm^2, was captured using a high-resolution video camera (Hamamatsu chilled CCD, model C5985) and 20x objective (Figure 1-1). RGCs were identified by the presence of fluorescence, their general morphology, as well as their soma sizes relative to other retinal neurons. The use of multiple criteria was necessary to avoid counting errors associated with label released from dying neurons and taken up by glia. The total number of RGCs counted in the 12 retinal images were divided by the total area of the retinae sample region to determine the mean density of labeled ganglion cells (neurons/mm^2) for each retina.

Statistics

All cell measurements are expressed as mean ± SD. Analysis of variance (ANOVA) with Bonferroni's test for multiple comparisons was used to compare

differences in mean RGC number between the normal, crush only, and crush + BDNF treatment groups. The level of significance used was p=0.05.

RESULTS

Using the Gel-foam-based labeling method described above, fluorogold labeled RGCs were seen distributed uniformly across the retina. Fluorescence was observed in the soma and some proximal dendrites of RGCs of the normal animals (Figure 1-2A). The labeling appeared homogeneous at low power (10x), but showed a punctate pattern when viewed at higher magnification (50x) (Figure 1-2B). The mean density of RGCs in the normal retinae was 1747±190 cells/mm^2.

One week following the crush, the mean density of ganglion cells in the affected eyes was 1201±7 cells/mm^2 ($p<0.05$, compared to normal), reflecting a 31% decrease. Application of BDNF (5μg) into the vitreal chamber at the time of the nerve crush promoted RGC survival (Figure 1-2D). The mean RGC density in the BDNF treated eyes was 1643±280 cells/mm^2, reflecting only a 6% reduction in ganglion cell density compared with normal ($p>0.05$). The change in mean RGC density expressed as a percentage of normal is shown graphically in Figure 1-3.

The superior colliculus represents a main postsynaptic target of RGC axons in the rat. To determine whether any RGCs maintained connections with the superior colliculus following the optic nerve crush, Fluorogold was applied to the superior colliculus in two rats post-crush. This resulted in only 3-5 retrogradely labeled cells in each retina (Figure 1-4).

DISCUSSION

The goal of this series of experiments was two-fold: 1) to establish that my approach to induce optic nerve injury could serve as a model for studying RGC degeneration, and 2) to verify that, in my hands, BDNF treatment is effective in promoting RGC survival following optic nerve damage.

The mammalian retina is composed of several different classes of neurons. The somata of photoreceptors are located in the outer nuclear layer, interneurons (including bipolar cells, horizontal cells, and amacrine cells) are located in the middle layer (inner nuclear layer), and RGCs are located in the ganglion cell (innermost) layer. However, there also is a group of amacrine cells located in the RGC layer. Since the soma sizes of these so called 'displaced amacrine cells' are similar to those of small RGCs in the rat, Fluorogold, a retrograde neuronal tracer, is used to selectively label the RGCs. Retrograde neuronal tracing is a technique often used to identify specific populations of neurons. The tracing material is applied to the site of axon terminals of desired neurons, which usually is located in a different area of the brain, and is taken up by endocytosis and transported retrogradely along the axons to fill the somata and dendritic processes of the parent cell. Specific visualization methods then are used to identify the neuronal population of interest. Fluorogold is a commonly used retrograde tracer. Cells labeled by Fluorogold can be examined without further processing, and unlike many fluorescent dyes, it resists photobleaching and is stable over a wide range of chemical (pH) and physical (light, temperature, time) conditions. When applied to the superior colliculus, a primary target area of RGC axons, Fluorogold labels RGCs uniformly across the retina

(Figure 1-2) and thus allows accurate assessment of the level of RGC death induced by optic nerve crush.

Damage to the optic nerve has been used previously to study the death of RGCs as well as various neuroprotection strategies. Among the different methods reported, transection of the nerve is used most widely. This model represents a worst-case scenario, however, since it involves severing all of the RGC axons. Glaucoma, on the other hand, is thought to result from gradual mechanical compression of the nerve. Therefore, crushing of the optic nerve is used here to better mimic RGC death in glaucoma, because it may not damage all the axons. Optic nerve transection in adult rats has been reported to result in the death of approximately 50% of the RGCs within 1 week [27,28]. By contrast, we found that optic nerve crush resulted in only a 31% loss of RGC after the same survival period. The result that more RGC survived crush injury than transection confirms that the damage caused by crushing is less severe than that caused by transecting. Furthermore, to examine if any RGC axons still have active connections with their targets after the optic nerve was crushed, I applied Fluorogold after, rather then before, the nerve crush. This resulted in the labeling of 3-5 cells per retina. Taken together, the data suggest that optic nerve crush caused a graded injury to the axons of retinal ganglion cells. A small population of ganglion cells maintains their connection with the target and has normal retrograde axonal flow. The majority (~69%) of the ganglion cells are injured and halted their axonal transport, but these cells are still alive as seen in the Fluorogold label first experiment. The most severely damaged axons (~31%), however, lead to the death of their parent cells.

One might argue that most of the RGCs in this study died due to ischemia, and not as a result of damage to the optic nerve. I think this is unlikely, since previous studies have shown that 30 minutes of complete central retinal artery occlusion in the rat produces no histological changes in the retina, and that only mild ischemic damage is present following 60 minutes of retinal ischemia [40,40-42]. Since the optic nerve crush used here did not exceed 15secs in duration, it is highly unlikely that ischemia played a role in causing RGC death in these studies.

The result that BDNF promoted RGC survival following optic nerve injury are in agreement with previous studies [19,25-28,30,31]. The mechanism of BDNF's protective effect, however, only has been partially understood. BDNF acts through its high affinity receptor TrkB, which has been located on RGCs [43-47]. Binding of BDNF with TrkB has been demonstrated to result in the interruption of apoptosis, or programmed cell death, in CNS neurons [48-52]. Apoptosis is a form of suicide in which the affected cell activates an internal program that leads to its degradation and removal without a significant negative effect on its neighbors. BDNF is known to prevent apoptosis in cultured cerebellar granule neurons [49,52] and cortical neurons [51] in vitro. It also has been shown to protect RGCs in vivo [19,25-31]. The protective properties of BDNF involve activation of extracellular signal-regulated kinase (ERK) pathway [53] and the phosphatidylinositol-3 (PI-3) kinase pathway [51]. More importantly, BDNF is known to block caspases, key enzymes in the apoptosis pathway [48,54]. These enzymes have been shown to induce apoptosis following optic nerve injury [55-57]. Thus it is not surprising to find that BDNF is effective in protecting RGCs after optic nerve damage.

In summary, these initial studies demonstrate that a controlled optic nerve crush produces a consistent, graded, level of retinal degeneration, and that this method is reasonable for evaluating the effects of different neuroprotectants. They also confirm that intravitreal application of BDNF promotes RGC survival following optic never damage in the small rat eye. However, if this drug is to be used as a neuroprotectant in glaucomatous eyes, it first is necessary to determine its potential neuroprotective effects in primate-sized eyes, where dose and diffusion differences might be limiting factors.

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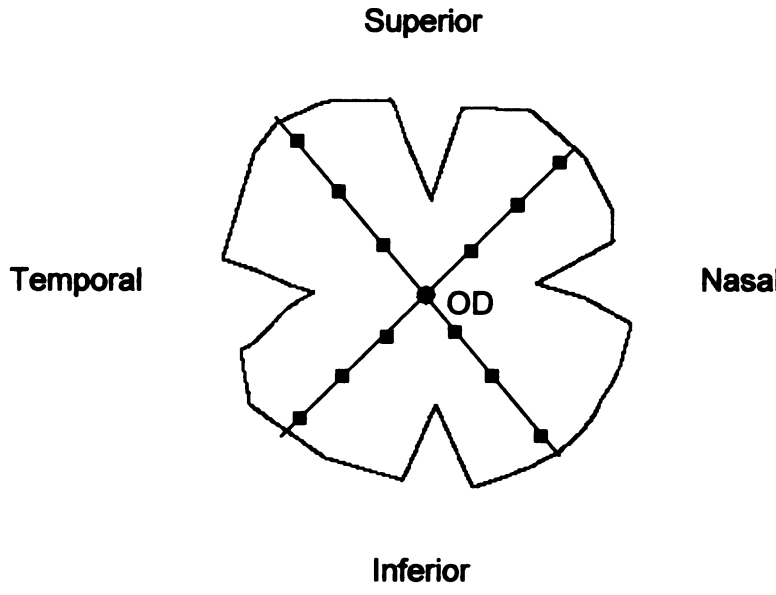

Figure 1-1. Schematic drawing showing the area sampled for counting the number of retina ganglion cells. Three rectangular microscopic fields each covering 41,125μm^2 were sampled in each retinal quadrant. OD: optic disk

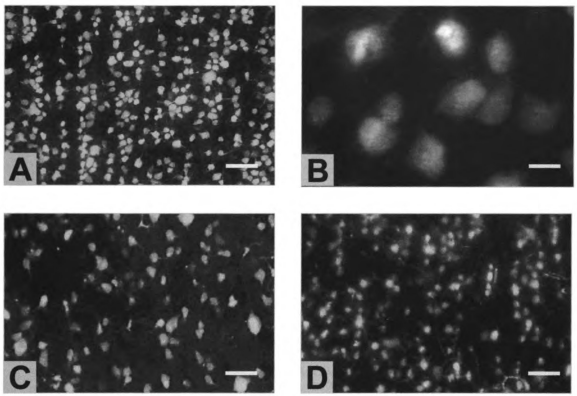

Figure 1-2. Retina whole mounts were prepared 7 days after Fluorogold was applied to superior colliculus, the main target of retinal ganglion cell (RGC) axons in the rat. RGCs were evenly labeled across the normal retina (A). Punctated appearance of the cells under higher magnification (B) is due to local accumulation of the fluorescent dye. Optic nerve crush caused a prominent loss of RGC (C) while brain-derived neurotrophic factor (BDNF) promoted the survival of RGC (D). Bar: 30 μm in A, C, and D. 10μm in B.

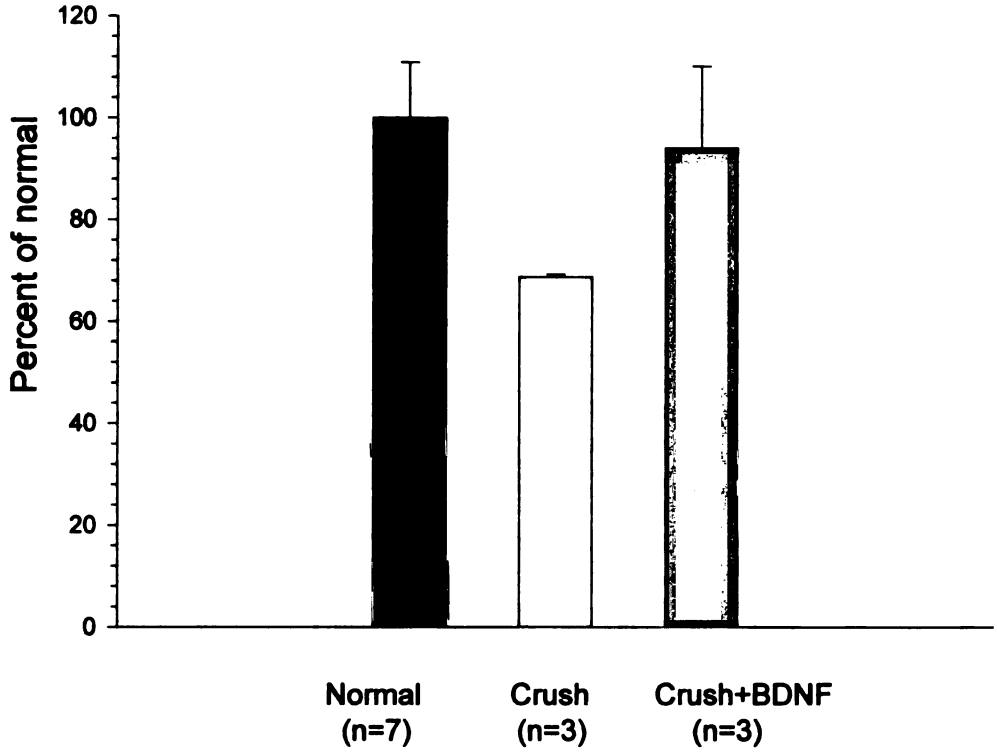

Figure 1-3. Counting of Fluorogold labeled retinal ganglion cells confirmed the reported effectiveness of BDNF in promoting RGC survival following optic nerve injury in rodents. Controlled optic nerve crush induced a 32% loss of RGC one week post the injury, while BDNF treated group showed only a 6% loss. Error bars are ± SD.

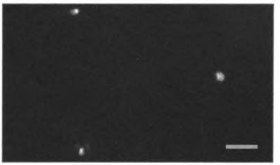

Figure 1-4. Rats first received optic nerve crush and then Fluorogold was applied to superior colliculus, the main target area of retinal ganglion cells (RGC) in the rat. The presence of RGC in this preparation demonstrated that some RGC axons still have active connection with their targets after optic nerve crush, suggesting that optic nerve crush caused a graded damage to the RGC axons. Bar: 30 μm.

Chapter II

Brain Derived Neurotrophic Factor Enhances Retinal Ganglion Cell Survival in Cats With Optic Nerve Damage *

INTRODUCTION

Primary open-angle glaucoma (POAG) is a disease of the visual system that results, in part, from pressure-induced damage to the optic nerve at the level of the lamina cribrosa (LC).[1-2] In addition to nerve fiber degeneration, the disease also is characterized by atrophy and a loss of ganglion cells from the retina itself.[3-8] Since previous work has shown that elevation of intraocular pressure disrupts axonal transport at the LC, the ganglion cell loss associated with POAG is considered to reflect, in part, a decrease in the level of trophic material these neurons receive from their target sources.[9-12]

Recent studies, using *in vivo* and *in vitro* techniques, have shown that neurons and glia within the mammalian retina contain receptors for different neurotrophic factors, and that direct application of these factors can influence the survival of injured ganglion cells.[13,14] In particular, studies of the rat visual system, have indicated that brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family of proteins, is highly effective in reducing the rate of axotomy-induced retinal ganglion cell death.[15-19] BDNF also has been shown to undergo both anterograde and retrograde axonal transport, and it has been implicated in reducing the retraction of axons after optic nerve lesion and

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* This Chapter is based on a paper accepted by *Investigative Ophthalmology & Visual Research*

promoting axonal regeneration following optic nerve injury.[20,21]

Based on these data, and a longstanding interest in different treatment strategies for reducing retinal ganglion cell degeneration in the glaucomatous primate eye, we initiated a series of experiments aimed at determining whether BDNF might also exert a neuroprotective effect on injured ganglion cells in the larger cat eye, where, compared with the small rat eye, drug dose and diffusion differences might be limiting factors.

MATERIALS AND METHODS

Subjects

Twenty-nine adult cats were used in this study. All were specific-pathogen free and weighed 3-4Kg. The cat was selected for use based on ease of manipulating its optic nerve, the well-defined morphologies of cat retinal ganglion cells,[22-27] and the similar vitreal volumes of the cat and primate eyes compared with that of the rat (~3-4ml vs ~50µl in the rat).[28, unpublished data] All procedures were approved by the Animal Use Committee at Michigan State University, and all adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgical Procedures

Initial anesthesia was achieved by placing the cat in a plexiglass chamber and introducing a mixture of 4% isoflurane (IsoFlo, Abbott Laboratories, Abbott, IL) and pure oxygen, delivered at 3l/minute. Each cat then was intubated, and anesthesia maintained using a 2.5-3.5% isoflurane/oxygen mixture (0.5l/min.). Analgesia and sedation consisted of an intramuscular injection of glycopyrrolate (0.05mg/kg; Ft. Dodge

Laboratories, Ft. Dodge, IA), and subcutaneous injections of torbugesic (0.2mg/kg; Butler, Columbus, OH) and acepromazine (0.04mg/kg; Butler). Hydration was maintained intravenously with sterile saline (0.9%). Heart and respiratory rates were monitored every 15 minutes. Body temperature was maintained at 37°C using a heating pad. The head was stabilized using a vacuum-activated, 'beanbag-like', restraining device (Olympic Vac, Olympic Medical, Seattle, WA). In 5 animals, the pupils were dilated with 1% tropicamide HCl (Mydriacyl, Alcon Laboratories, Ft. Worth, TX) and contact lenses containing 1-2 drops of 0.5% proparacaine HCl (Alcaine, Alcon) were placed on the eyes. Pre- and post-surgery fundus photographs of the retinal blood vessels were obtained using a Topcon TRC-50 fundus camera (Topcon, The Netherlands). Additional fundus photographs were obtained at the time of sacrifice.

Using sterile procedures, the bone overlying the left frontal sinus was removed to expose the roof of the bony orbit. All openings to the frontal sinus then were sealed with bone wax. This avoided disturbing the cat's olfactory senses, which can result in a severe loss of appetite. A fine-tipped scalpel blade was used to make an opening in the dorsal surface of the orbit. Careful blunt dissection of the overlying tissues exposed the optic nerve without disturbing the nerve sheath or retinal artery. The optic nerve (ON) was stabilized with a hook, and a smooth-faced bulldog clamp that exerts ~1,024 grams of force was place on the nerve for 15 seconds at a distance 2-3mm behind the globe. The bone wax plugs then were checked, the frontal sinus was packed with Gelfoam (Upjohn, Kalamazoo, MI) soaked with sterile saline, and the overlying skin sutured. The contact lenses were removed, and the eyes treated with sterile ophthalmic ointment. Following removal of the intubation tube, each animal was monitored until it was able to move

about freely and feed. Post-operative pain medication (torbugesic, 0.2mg/Kg) was provided as needed.

BDNF Injections

Single or multiple intravitreal injections (15, 30, 60, or 90μg @ 1μg/μl) of sterile recombinant BDNF (Regeneron Pharmaceuticals, Tarrytown, NY) were made into the left (ON crush) eye of 19/29 cats. Of the remaining 10 cats, 3 did not receive any surgical procedures, 5 received a unilateral optic nerve crush but no treatment, 1 received an ON crush and an intravitreal injection of 60μl of sterile water, and 1 received an intravitreal injection of sterile water, but no ON crush. Three animals that received 90μg injections of BDNF also received, for 1 week, daily intraperitoneal injections (35mg/Kg) of the nitric oxide synthase blocker, L-NAME (N-ω-nitro-L-arginine-methylester), since nitric oxide has been implicated to be at least partially responsible for RGC death. All BDNF injections were made immediately following the optic nerve crush. In most cases the injections were made through the opening in the frontal sinus at a point ~5mm posterior to the ora serrata. Three animals received a second intravitreal injection of the same dose 4 days post-crush (Table 1). For these injections, which were made just posterior to the ora, the animals were anesthetized with ketamine HCl (10mg/kg) and the eyes treated with Alcaine. All injections were made using a Hamilton syringe with a 30 ga. needle. Care was taken to ensure that the complete bevel of the needle was within the vitreal chamber, but that it did not hit the lens.[29] Intraocular injections were made over a 1 minute period, with the needle left in place for an additional 30 seconds to allow for diffusion of the drug away from the injection site.

Survival Period and Tissue Processing

Following a 7 day survival period, 24/29 of the animals were anesthetized deeply with pentobarbital sodium (50mg/kg), and perfused transcardially with 0.5l of saline (0.9%) followed by 2l of a solution containing 1.5% paraformaldehyde and 2.0% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.4). One week was selected as the baseline survival period because initial cell measurements showed that this time period resulted in a consistent, but not overly severe, change in ganglion cell number (~50% decrease) in the untreated eye (Table 1, Figure 2-5). Following overnight post-fixation, the retinae were dissected, whole-mounted onto subbed glass slides, dehydrated, and stained with cresyl violet. Care was taken during mounting to make the relief cuts in superior temporal retina as shallow as possible. This avoided any distortion in the region from which the ganglion cell samples were obtained.

Ganglion Cell Measurements and Identification

Retinal ganglion cells from normal, untreated, and BDNF-treated eyes were compared using a computer-based imaging system. The region selected for quantitative analysis occupied 1.72mm^2, and was located 3mm above and 1.5mm temporal to the area centralis (Figure 2-1). This region was chosen because of the relatively constant size and density of ganglion cells in this location of the cat retina.[22] A stage digitizer (AccuStage, Shoreview, MN) was used to properly orient[30] each retina on the microscope, and to standardize the starting point and stage movements used for cell sampling. From the starting point, 42 digital images (41,000μm^2/image) were obtained systematically using a

high resolution video camera (Hamamatsu chilled CCD, model C5985) and 40x objective. The retinal images were collected as 3 dorsal-ventral passes composed of 14 images each. Double counting was avoided by separating each sample column horizontally by 500um, and vertically by using the previous image as a reference. Cell size, density, and number were determined directly from the digital images using image analysis software (Image Pro Plus, Medial Cybernetics). Neurons were classified as ganglion cells based on the criteria of Stone.[24,25] In brief, they had to display a distinct nucleus and nucleolus, and have a clear ring of cytoplasm completely surrounding the nucleus. Although this conservative approach most likely underestimated the number of small ganglion cells, it also reduced the number of non-ganglion cells included in the measurements.[26,27] Based on these criteria, our estimates of ganglion cell density, as well as the proportions of small, medium, and large ganglion cells in the normal sample region were comparable to those reported by Stone.[24]

Statistical Analysis

All data are presented as means ± 1SD. Differences in mean cell number, size, and density were compared using one-way analysis of variance (ANOVA), combined with the Bonferroni adjustment for multiple comparisons (SPSS, Chicago, IL). Paired comparisons of cell size distributions were made using the Kolmogorov-Smirnov test (SPSS) for two independent samples. In all cases, p=0.05 was used as the level of significance.

RESULTS

Effects of ON Crush on Retinal Vasculature

In all cases, optic nerve damage was achieved by placing a small bulldog clamp on the optic nerve 2-3mm behind the left eye for 15 seconds. Fundus photographs obtained pre- and post-surgery, as well as following the 1 week survival period for five randomly-selected animals, indicated that this brief period of pressure had no adverse effect on the vasculature of the inner retina.

Cellular Changes in Untreated Retinae

While brief optic nerve compression did not compromise the retinal vasculature, it did result in a significant loss of ganglion cells (~50%) from the retina within the 1 week test period. Neurons undergoing atrophic changes were identified by their irregular shape, pale-staining cytoplasm, clumped chromatin, and displaced nuclei (Figures 2-2). Although no degenerating neurons were observed in normal retinae, systematic examination (250x) of the sample region in animals receiving an ON crush but no treatment with BDNF revealed a high density of atrophic profiles (~26.9 profiles/mm^2) in these retinae.

The data presented in Figure 2-3 compare the cellular morphology of the normal cat retina with that from cats that received a unilateral ON crush, a 1 week survival period, and no BDNF treatment. For the 24 normal retinae examined, we found the sample regions to contain an average of 539±48 ganglion cells, with a mean population soma size of 411±47μm^2, and a mean cell density of 313±28 neurons/mm^2 (Table 1.).

Twenty six percent of the ganglion cells in the sample region had small (65-300μm^2) somata, 68% had medium-sized cell bodies (301-800μm^2), and 6% contained large (800-2000μm^2) somata.

One week following optic nerve crush, the mean number of ganglion cells within the sample region decreased to 263±39 neurons, a reduction of 51.2% (Table 1., Figure 2-3 & 2-5). Mean soma size decreased 23%, to 316±57μm^2, and cell density now was only about one-half normal (151±28 neurons/mm^2). The percentage of neurons with large cell bodies decreased from 6% to 3.2%, while those with medium-sized somata decreased from 68% to 40.6% of the total population. While the proportion of small ganglion cells within the sample region more than doubled (56.2% vs 26%), this increase was due primarily to the loss of large (from 98 to 25) and medium-sized neurons (1099 to 316), and not a significant increase in the number of small neurons (420 to 438) due to cell shrinkage. These cellular changes are reflected in the cell size histograms of Figure 2-3, which show a clear decrease in the number of large and medium-sized ganglion cells, and a shift toward smaller soma sizes. The untreated retinae also showed a clear increase in the number of glial cells (c.f. Normal vs Untreated, Figure 2-3). Despite these changes, however, most surviving ganglion cells retained their round-oval shape, and well-defined nucleus and nucleolus.

Cellular Changes in Sham and BDNF-treated Retinae

Single injections

The photomicrographs and cell size histograms in Figure 2-4 compare the cellular morphology of the 24 normal retinae with that from the 12 animals (3/treatment

condition) that received intravitreal injections of BDNF at the time of nerve crush. In all cases, the BDNF-treated animals were examined following a 1 week survival period.

Injecting either sterile water (60µl) or 15µg of BDNF into the vitreal chamber at the time of the nerve crush had little or no beneficial effect. The retinae from these cats did not differ qualitatively or quantitatively from those of cats that received an optic nerve crush, but no BDNF treatment (Table 1., Figure 2-3). Both the untreated and the 15µg BDNF-treated retinae contained many microglia and a high density of atrophic profiles (~28profiles/mm^2). Both also showed comparable ganglion cell loss (51% and 48%, respectively). The density of ganglion cells in the sample regions of the untreated and 15µg-treated retinae were 151 neurons/mm^2 and 163 neurons/mm^2, about one-half the cell density measured in the normal retinae (313 neurons/mm^2). Cell size measurements (Figure 2-5B) indicated ~2-fold greater decrease in mean soma size in the untreated eyes (23%) than in those receiving 15µg of BDNF (11%), but neither reduction was significantly different from normal. The sample region in the BDNF-treated eyes contained a lower percentage of small ganglion cells (46.8% vs 56.2%), and higher percentages of medium (48.2% vs 40.6%) and large (5.2% vs 3.2%) ganglion cells compared with the untreated eyes. Although this difference is indicated by a reduced amount of skew in the cell size histogram of the 15µg BDNF vs untreated animals (c.f. Figure 2-3 & 2-4), the cell size distributions for both groups of animals were statistically different from normal.

Intravitreal treatment with 30µg of BDNF at the time of nerve crush resulted in a significant improvement in the number and appearance of surviving ganglion cells (Figure 2-4 & 2-5). Neurons in these retinae had well-defined membranes, uniformly

distributed Nissl substance, and a clear nucleus and nucleolus. The mean percent difference in ganglion cell number between the normal and treated eyes for these animals was 19%, indicating a survival level of 81%. This represents a significant saving of ganglion cells compared with the untreated and 15μg BDNF-treated eyes (49% and 52%, respectively). Although ganglion cell density in the sample region of these animals was only 81% of normal, it was 60% higher than that measured in the untreated and 15μg BDNF-treated retinae. The density of atrophic profiles in the 30μg BDNF-treated animals was only 4.6 profiles/mm^2, significantly lower than the ~28 profiles/mm^2 measured in the untreated and 15μg BDNF-treated retinae. Ganglion cells in the 30μg BDNF-treated eyes were ~5% smaller than normal (389μm^2 vs 411μm^2), but as a population had a mean soma size that was 13-23% greater than that of the untreated (316μm^2) and 15μg BDNF-treated (367μm^2) eyes. These differences were not statistically significant. The percentages of ganglion cells within the sample region with small, medium, and large somata in the 30μg BDNF-treated eyes were 39.9%, 53.9% and 6.2%, respectively. This represented an increase in the proportions of cells with large and medium-sized somata, which resulted in a broadening of the cell size distribution compared with normal. The two distributions, however, were not statistically similar.

Increasing the dose of BDNF to either 60μg or 90μg also resulted in a significant improvement in the appearance and number of surviving ganglion cells when compared with either no treatment or treatment with 15μg of BDNF. Like the 30μg BDNF-treated animals, the sample regions of these eyes contained low densities of atrophic profiles (6.6 and 1.3 profiles/mm^2, respectively). However, unlike the 15μg and 30μg BDNF-treated animals where increased levels of BDNF produced increased numbers of surviving

ganglion cells, here ganglion cell number decreased with the application of higher doses of neuroprotectant (30μg: 81%; 60μg: 77%; 90μg: 70%). The mean number of ganglion cells in the sample region of the 60μg BDNF-treated eyes (414) was significantly greater than that measured in the untreated (263) and 15μg BDNF-treated eyes (281), but the mean number measured in the 90μg BDNF-treated eyes (378) was not (Figure 2-5A, Table 1.). Overall, the ganglion cells in the 60μg-treated eyes were ~16% smaller than normal (345μm^2 vs 411μm^2), while those in the 90μg-treated eyes were slightly larger than normal (420μm^2). The sample region in the 60μg-treated eyes showed a slightly lower than normal proportion of ganglion cells (4.2% vs 6%) with large somata and a higher than normal proportion (53% vs 26%) with small somata. The cell size distributions indicated a continued reduction in the number of ganglion cells with medium-sized somata. By contrast, the proportions of ganglion cells with small, medium, and large somata in the eyes treated with 90μg of BDNF were almost identical to those measured in the normal eyes (30.5% vs 26%; 64.2% vs 68%; 5.4% vs 6%). Mainly this was due to the increased survival of medium-sized ganglion cells. Nevertheless, the cell size distributions for both the 60μg and 90μg remained statistically different from normal.

One noticeable difference between the 30μg, 60μg, and 90μg BDNF-treated retinae was a clear increase in the number of inflammatory cells (e.g. macrophages, leukocytes, etc.) present with increased levels of the drug (Figure 2-4 & 2-6). In most cases the inflammatory cells were distributed near blood vessels, or scattered randomly across the retina. However, in some areas these cells appeared to be clustered over specific neuronal profiles (Figure 2-6B).

Multiple BDNF injections

Three cats with unilateral optic nerve crush received dual injections of BDNF. In all animals, the first injection was made at the time of the optic nerve crush and the second injection 4 days post-crush. Two cats received double injections of 30μg of BDNF, while the third cat received multiple 60μg BDNF injections. In all cases, the percentage of surviving ganglion cells was comparable to that of a single injection given at the time of the nerve crush (30μg: 81% vs 84%; 60μg: 77% vs 76%). Similarly, no differences were seen with respect to single vs multiple injections and mean soma size (30μg: 389μm^2 vs 408μm^2; 60μg: 345μm^2 vs 345μm^2) or ganglion cell density (neurons/mm^2) within the sample area (30μg: 254 vs 264; 60μg: 244 vs 237).

Combined BDNF and L-NAME Injections

Three cats that received high doses of BDNF (90μg) also were treated during the 1 week survival period with daily injections of L-NAME, a nitric oxide synthase specific inhibitor (35mg/Kg/day). While treatment with L-NAME eliminated the inflammatory response induced by the high levels of BDNF, it did not result in a significant change in the size, number, or density of ganglion cells measured when compared with animals that received 90μg injections of BDNF alone (size: 315.3±32.6μm^2 vs 420±64μm^2; number: 383±62.9 cells vs 378±31cells; density: 223±36.4 neurons/mm^2 vs 219±18 neurons/mm^2).

DISCUSSION

The primary goal of this study was to determine whether BDNF, a well-known neuroprotectant in the small rat eye, might also serve as an effective neuroprotectant in primate-sized eyes, where dose and diffusion differences may be limiting factors. The importance of these data derives from their potential use in the treatment of retinal diseases, and in particular glaucoma, where a reduction in target-derived neurotrophin levels has been implicated as playing a role in retinal ganglion cell death.[11,12]

In agreement with previous studies in the rat,[15-19] our data show that intravitreal application of BDNF also can enhance retinal ganglion cell survival in cats following optic nerve injury. This result was not unexpected, as ganglion cells in the cat retina (unpublished data), like those in the rat and other species,[31-33] express TrkB, the high affinity BDNF receptor. However, in contrast with the rat where relatively small amounts of BDNF (~0.5μg-5μg), and sometimes vehicle solution alone, have been shown to promote ganglion cell survival, we found that in the larger cat eye vehicle solution alone had no beneficial effect, and that ~30μg of BDNF was needed to achieve a significant level of neuroprotection. While this amount of drug might appear excessive, when one takes into consideration that the vitreal chamber of the cat eye is ~60-fold larger than that of the rat (3ml vs 50μl), the effective dose for these different eyes is about the same (~0.01μg BDNF/μl of vitreal volume).

Increasing the amount of BDNF injected above 30μg resulted in a decrease, rather than increase, in ganglion cell survival (Figure 2-5A). Eyes receiving 30μg of BDNF

showed the highest level of survival (81%), while those receiving 60μg and 90μg showed progressively fewer surviving cells (77% and 70%, respectively). A similar dose-related limitation in BDNF effectiveness has been reported in the rat retina,[34] as well as in other areas of the CNS.[35-37] While many factors might be involved, recent work has focused on two specific mechanisms. The first concerns BDNF-induced nitric oxide (NO) neurotoxicity,[36, 38-40] and the second involves BDNF-induced down-regulation of the TrkB receptor.[35, 41-43] Nitric oxide is a relatively ubiquitous molecule that modulates a number of different physiological processes. Typically, NO is localized in a tissue by immunocytochemical recognition of its synthesizing enzyme, nitric oxide synthase (NOS). Of the different isoforms, neuronal NOS (nNOS) and inducible NOS (iNOS) have been studied most completely in the retina. Species differences aside, there is good evidence that nNOS is found in all 5 of the major cell types of the vertebrate retina (ganglion, amacrine, bipolar, horizontal and photoreceptor), and iNOS is associated primarily with Müller cells and microglia.[38,39,44,45] Recent studies in the rat have shown that both nNOS and iNOS activity are elevated following optic nerve section and/or intravitreal injection of BDNF.[38,39] That increased NOS activity affects retinal ganglion cell survival is indicated by the neuroprotective action of concurrent administration of NOS inhibitors.[34, 38,39]

In addition to BDNF-induced iNOS activation in microglia, it also has been hypothesized that BDNF induces iNOS activity in immune-competent cells.[38] Both of these mechanisms are relevant to the present study, where ON crush produced an increase in the number of microglia (Figure 2-3 & 2-4) and high doses of BDNF (90μg), with ON crush or alone, generated a strong inflammatory response within the retina (Figure 2-4 &

6). Surprisingly, we found that treatment with the NOS-inhibitor L-NAME blocked the BDNF-induced inflammatory response, but did not enhance ganglion cell survival, a result in direct contrast with that obtained in the rat. While it is possible that differences in dose (50mg/Kg/day vs 35mg/Kg/day)[38] and route of administration (intravitreal vs intraperitoneal)[39] are the cause of this variation, the fact that the retinae of the L-NAME treated cats appeared normal suggests that iNOS derived from BDNF-activated immune cells was not a limiting factor. It does not rule out, however, incomplete blockade of other NO sources.

BDNF exerts its influence on ganglion and other cells in the retina via TrkB receptors.[46,47] There are two types of TrkB receptor, full length and truncated. The basic difference between the two is that the truncated form shows some amino acid residue variations and lacks the cytoplasmic tyrosine kinase domain. Neurotrophin binding to the extracellular domain of the full length receptor induces phosphorylation of tyrosine residues within the cytoplasmic domain. Downstream of the phosphorylated internal domain are several intracellular signaling pathways, and activation of these pathways has been shown to regulate gene expression related to cell death and survival.[48-52] Recent studies have demonstrated that continuous application of BDNF to the brain or cultured neurons results in a decrease in TrkB receptor protein and/or mRNA,[35, 41-43] and we have found this also to be true in the rat retina.[53] A single injection of BDNF (5μg) produces ~ 96% decrease in retinal TrkB protein over the first 24 hours. Recovery is slow, achieving only ~31% of normal at 14 days post-injection. Studies employing chimeric structures of the TrkA and TrkB receptors indicate that a short sequence in the juxtamembrane region of the cytoplasmic domain is responsible for neurotrophin-induced down regulation of

the TrkB receptor.[42] Based on these data, it is reasonable to hypothesize that BDNF-induced down-regulation of the TrkB receptor might also have played a role in limiting drug effectiveness in the present study. In addition, it might also have been responsible for our failure, and that of others, to achieve enhanced ganglion cell survival via the administration of multiple injections of BDNF. Given the rapid receptor down-regulation and long recovery time[53], it is not surprising that our administration of a second BDNF injection just 4 days post-crush did not increase ganglion cell survival over that seen with the initial treatment. DiPolo et al.,[19] did not find a similar decrease in TrkB effectiveness (until ~10 days post-axotomy) with prolonged delivery of BDNF, however, this result might reflect a positive side to their approach. By transfecting retinal Müller cells to produce and release BDNF, their method of drug delivery was much less invasive than that used here and in the other rat studies. In addition, the relatively slow delivery of BDNF in their retinae might have allowed adjacent truncated TrkB receptors to better buffer the concentration of drug within the eye,[54,55] thereby preventing BDNF-induced rapid degradation of the full-length TrkB receptor.

The cell size measurements (Figure 2-3 & 4) indicate that although both large and medium-sized ganglion cells are affected, ON crush has a much more severe effect on the medium-sized cells. One week following ON crush and no BDNF treatment, both populations of neurons showed ~75% reduction in ganglion cell number within the sample area. However, because medium-sized ganglion cells comprise a much larger proportion of all ganglion cells in the cat retina,[22-27] the number of medium-sized ganglion cells lost from these retinae was about 10-fold greater than that of large ganglion cells (783 medium vs 72 large). Shrinkage of medium-sized cells did not appear

to be a significant factor, as there was only a 4% increase in the number of small ganglion cells in these animals. A rapid and severe loss of medium-sized ganglion cells is consistent with other studies of optic nerve damage in the cat,[56-58] including elevation of IOP.[59] A similar selective loss of medium-sized cells also has been reported in the avian retina with experimental glaucoma.[60] While these results appear to be in contrast with human glaucoma, where it generally is thought that large ganglion cells are most susceptible, anatomical[7] and physiological[61,62] evidence indicate that small and medium-sized ganglion cells also can be affected severely in the glaucomatous human retina.

The primary effect of the BDNF treatments was to restore balance to the proportions of small, medium, and large sized ganglion cells within the sample region. Treatment with 30μg of BDNF saved the largest number of ganglion cells, including the highest number of large ganglion cells. The increase in medium, and particularly small ganglion cells, might reflect the increased ability of the BDNF at this dose to block the rapid loss of medium-sized cells, but not maintain their normal size. Increasing the amount of BDNF injected to 90μg caused a reduction in the number of large and small ganglion cells, but produced the largest saving of medium-sized neurons. This resulted in a normal balance in the cell proportions. Although more cell specific studies are needed, the data suggest differential sensitivities of large and medium-sized ganglion cells to intravitreal application of BDNF. Large ganglion cells appear to respond well to low doses, but their survival is limited by high doses. Medium-sized cells appear to respond less well to low doses, but do not show a decline with high levels of drug application. This differential effect might reflect differences in the number and type (full length vs truncated) of TrkB receptors present on large vs medium ganglion cells, or it might

reflect differences in retinal circuitry. For example, the close association of large ganglion cells with amacrine cells, which also contain TrkB receptors and are capable of producing NO, might be a disadvantage to these neurons in the presence of high levels of BDNF. Ongoing studies are aimed at isolating the potential differential influences of BDNF on the various classes of cat ganglion cells.

In summary, the data presented here indicate that BDNF is a suitable neuroprotectant for use in primate-sized eyes. The best intravitreal dose for short-term treatment, which might be sufficient when combined with a reduction in IOP, appears to be ~0.01μg BDNF/μl vitreal volume. Improving the long-term neuroprotective effectiveness of BDNF will require a better understanding of the differential effect the drug has on different classes of ganglion cells, as well as the relation between these neurons and other BDNF-sensitive elements of the retina.

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TABLE 2-1. Summary of Cell Number, Size and Density Measurements

		Normal	No Treatment			BDNF Treatment					
			1wk	2wks	4wks	15ug	30ug	60ug	90ug	30ugx2*	60ugx2*
Cell Number	Mean	539	263 (49%)	123 (23%)	50 (9%)	281 (52%)	438 (81%)	414 (77%)	378 (70%)	454 (84%)	407 (76%)
	SD	48	39	0	0	41	42	92	31	5	0
	N	24†	3	1	1	3	3	3	3	2	1
Cell Size (um^2)	Mean	411	316 (77%)	476 (116%)	443 (108%)	367 (89%)	389 (95%)	345 (84%)	420 (102%)	408 (100%)	345 (84%)
	SD	47	57	0	0	51	130	14	64	9	0
	N	24	3	1	1	3	3	3	3	2	1
Cell Density (N/mm^2)	Mean	313	151 (48%)	72 (23%)	29 (9%)	163 (52%)	254 (81%)	244 (78%)	219 (70%)	264 (84%)	237 (76%)
	SD	28	25	0	0	24	25	58	18	3	0
	N	24	3	1	1	3	3	3	3	2	1

* BDNF injections on day 0 and 4

† Number of eyes examined: includes fellow eyes from crush only and BDNF-treated animals, but not sham or L-NAME treated

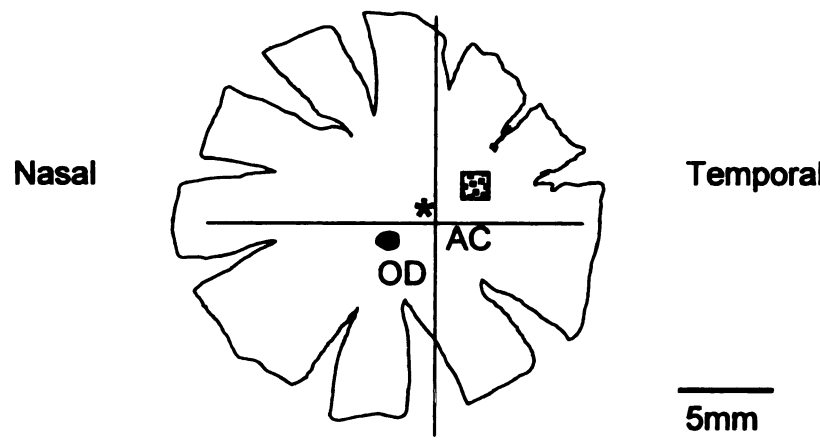


Figure 2-1. Schematic drawing showing the approximate location of the retinal sample area (stippled) for the left retina. The area centralis (AC) and optic disc (OD) were used as references to properly orient the retina.

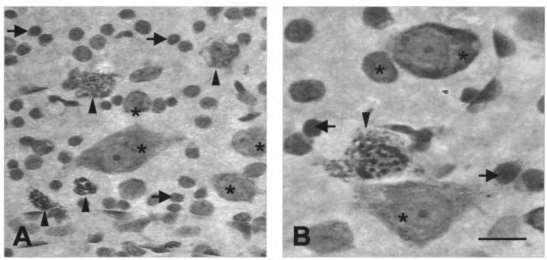


Figure 2-2. Low (A) and High (B) power photomicrographs showing examples of normal (stars) and degenerating (arrowheads) ganglion cells following optic nerve crush. Examples of glial cells are indicated by arrows. Atrophic neurons are recognized by their irregular shape and clumped chromatin. Scale = 30mm (A); 15mm (B).

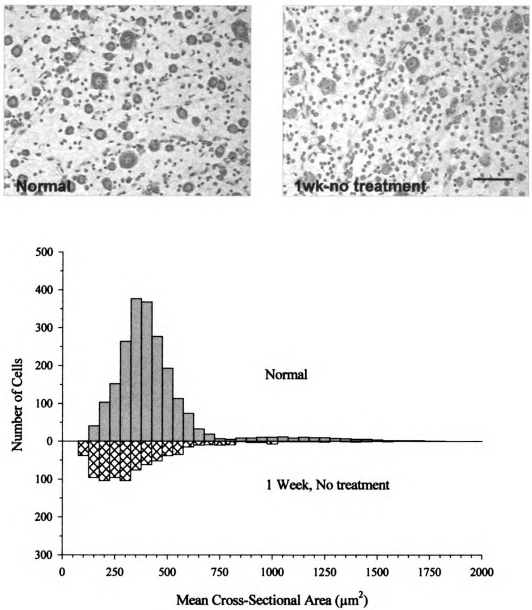


Figure 2-3. Photomicrographs and cell size histograms comparing the cellular morphology of the normal retina with that from cats that received an ON crush but no BDNF treatment. ON injury resulted in a significant loss of medium- and large-sized ganglion cells, and an increased level of gliosis. The data are based on 3 animals under each condition. Scale = 50μm.

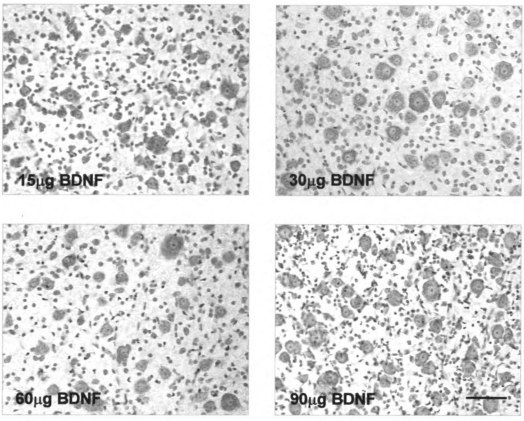


Figure 2-4. A. Photomicrographs comparing the cellular morphology of the normal retina with that of cats that received an ON crush and different levels of BDNF treatment. The highest percentage of surviving ganglion cells were measured following 30mg injections. Higher doses resulted in inflammation and reduced ganglion cell survival. The data are based on 3 animals under each condition. Scale = 50μm.

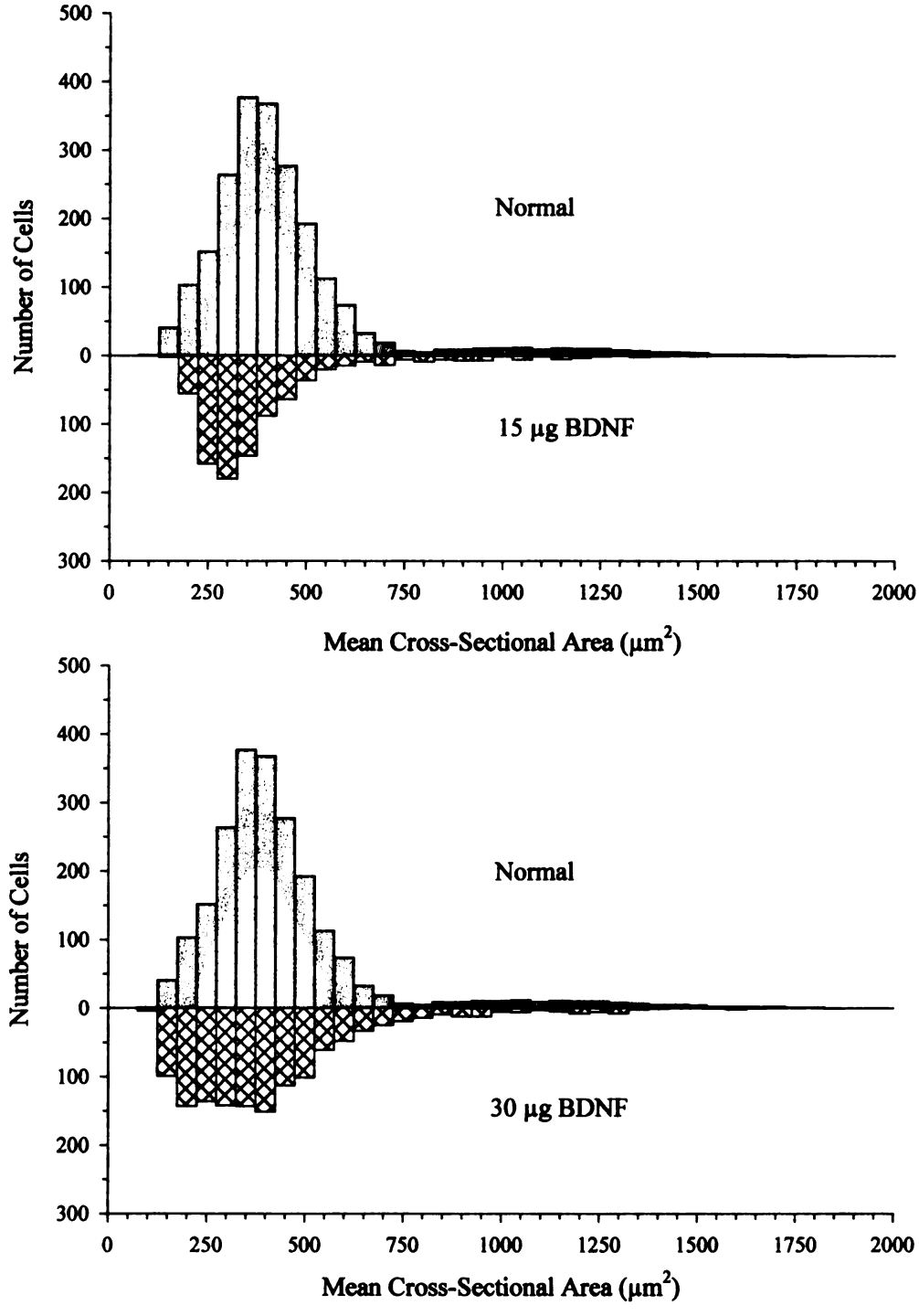


Figure 2-4. B. Cell size histograms comparing the cellular morphology of the normal retina with that of cats that received an ON crush and different levels of BDNF treatment. The highest percentage of surviving ganglion cells were measured following 30mg injections. Higher doses resulted in inflammation and reduced ganglion cell survival. The data are based on 3 animals under each condition. Scale = 50µm.

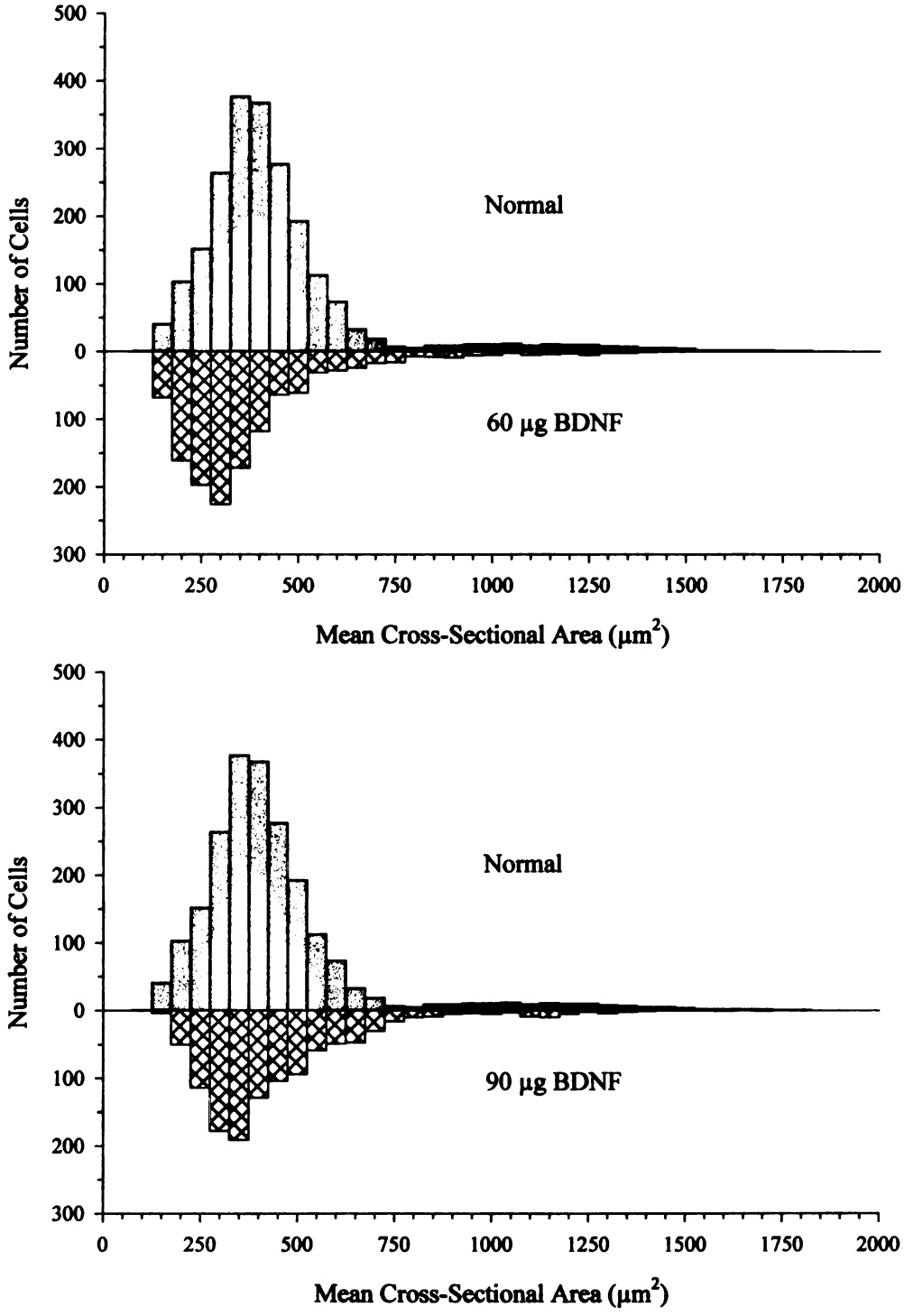
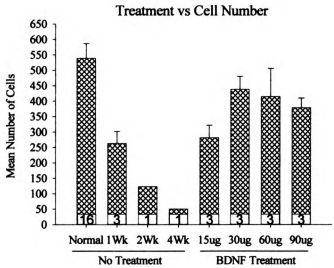


Figure 2-4 B (cont'd)

A



B

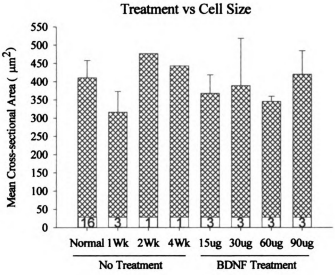


Figure 2-5. Summary histograms comparing the changes in cell number and size following different BDNF treatment paradigms. Eyes receiving 30mg and 60mg injections of BDNF showed significantly greater ganglion cell survival than the untreated or 15mg treated eyes. Values are the number of eyes examined under each condition. Error bars are ± SD.

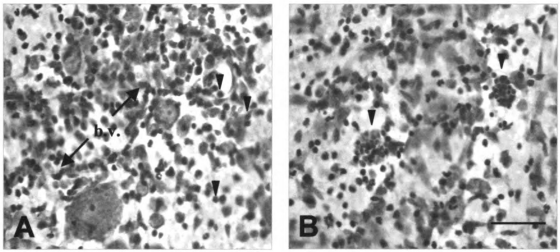


Figure 2-6. Photomicrographs showing the inflammatory response produced by injection of 90mg of BDNF. Most inflammatory cells (A, arrow head, macrophages or leukocytes) were either randomly distributed or associated with blood vessels (A, arrows). Others were clustered over unidentified retinal profiles (B, arrowheads). Scale = 50μm.

Chapter III

Temporal Changes in the Expression of Glia Fibrillary Acidic Protein (GFAP) and Glutamine Synthetase in the Rat Retina Following Optic Nerve Damage and Brain-Derived Neurotrophic Factor (BDNF) Application

INTRODUCTION

The mammalian CNS is composed of two general cell types, neurons and glia. While neurons provide the basis for complex electrophysiological activities, the primary role of glia is support. In addition to providing structural integrity to the nervous system, glia also have been shown to participate in neurotransmitter metabolism, to serve as modulators of synaptic transmission, and to play a role in maintaining a homeostatic environment for neurons.

In the retina, the primary type of glia is the Müller cell. Müller cells are bipolar-shaped cells that have their cell bodies located in the central, inner nuclear layer (INL) of the retina. The processes of Müller cells extend to both the inner and outer surfaces of the retina. At the outer level of the retina the endfeet of Müller cells form tight junctions with each other, and give rise to the outer limiting membrane. Similarly, the inner endfeet of Müller cells form the inner limiting membrane at the vitreal surface. The endfeet of Müller cells are relatively complex, and interdigitate with the photoreceptors of the outer retina and the ganglion cells of the inner retina. The spatial arrangement of Müller cells is such that they divide the retina into distinct compartments, the microenvironment of

which they control via their voltage-gated channels (e.g. inward rectifying potassium channels), neurotransmitter receptors (e.g. NMDA, GABA, and dopamine receptors), and glucose transporter system (reviewed in ref [1]).

In the previous set of experiments I demonstrated that BDNF is capable of reducing, but not reversing, retinal ganglion cell death following optic nerve injury. The goal of this set of experiments was to determine whether Müller cells might influence the neuroprotective effects of BDNF, and whether the inability to reverse cell death completely might be due, in part, to a decrease in the number of Müller cells, or their ability to respond to the injury. To test this hypothesis, the temporal expression of two glial-specific proteins was examined following optic nerve crush alone, and in conjunction with the application of BDNF. The two proteins examined were glia fibrillary acidic protein (GFAP) and glutamine synthetase (GS). GFAP is a 51kDa intermediated filament protein that is found in the Müller cell endfeet and processes. Although the functional significance of GFAP remains unclear, the fact that it is up-regulated by glial cells in response to injury has lead to its acceptance as an indication of an ongoing injury response. Glutamine synthetase is found in the Müller cell soma. In the rat, GS is an octamer with 8 identical 45kDa subunits. GS plays an important role in controlling the level of extracellular glutamate [2], which if left unchecked, can be excitotoxic to ganglion cells [3]. In brief, Müller cells take up the excess glutamate and via GS convert it to glutamine. They then release the glutamine into the extracellular space, where it is taken up by ganglion cells and reused to synthesize glutamate [1].

A decrease in either GS or GFAP levels following optic nerve damage might signal either a decrease in Müller cell numbers, or an inability of these critical support

cells to perform their normal maintenance role. These data are important for understanding the extent to which retinal ganglion cell death results from events not related to direct damage to the neurons themselves.

MATERIALS AND METHODS

Animals and Surgical Procedures

Adult Sprague-Dawley rats weighing 250-300g were obtained and maintained as describe in Chapter 1. Surgical procedures concerning the optic nerve crush and BDNF injection also were the same as those described previously.

Immunohistochemistry

Adult rats that received a unilateral optic nerve crush were allowed to survive for either 1 day (n=3), 3 days (n=3), 7 days (n=3) or 14 days (n=3). Since unilateral damage to one eye has been shown to result in the up-regulation of injury-responsive genes in the contralateral eye [4], retinae from three normal rats were used as the control tissue for these studies. Following their respective survival periods, all animals were anesthetized deeply with Nembutal (>35mg/kg) and perfused transcardially with 150ml of saline followed by 300ml of a solution containing 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The eyes then were removed and post-fixed for an additional hour at room temperature. The globes were bisected, and the posterior eye cups containing the retinae soaked in 20% sucrose in 0.1M PB at 4°C. The sucrose-saturated tissue then was embedded in Histo Prep (Fisher Chemical, Fair Lawn, NJ). For histological analysis, the

eye cups were sectioned at 20μm using a cryostat, and the sections were mounted onto subbed slides. To visualize GFAP, the sections first were incubated with CAS™ solution (Zymed, San Francisco, CA) to block non-specific binding for 1hr at room temperature (RT). Incubation in primary polyclonal anti-GFAP antibody (1:1000, 4°C, overnight, DAKO, Carpinteria, CA) was followed by incubation in a secondary goat-anti-rabbit IgG (1:200, 30min, RT, Vector, Burlingame, CA). Texas Red Avidin D (1:2000, 20 min, RT, Vector, Burlingame, CA) was used for the final visualization. Similarly, GS was detected by first incubating the retinal tissue with the CAS™ blocker (1hr, RT), followed by treatment with a monoclonal anti-GS antibody (1:1000, 4°C, overnight, Chemicon, Temecula, CA). The sections then were incubated in horse-anti-mouse IgG (1:200, 30min, RT, Vector). A diaminobenzidine staining kit (Scytek, Logan, UT) was used to visualize the location of GS within the tissue. For all procedures, the sections were washed 3 times (5min each) in 0.01M phosphate buffered saline between each step.

Counting of Glutamine Synthetase Positive Cell

The number of GS positive cells was counted using a microscope and 20x objective. Three adjacent retinal cross-sections from each rat were used, and a total length of 2,240μm of the inner nuclear layer was sampled on each section. Differences between normal and experimental animals were derived based on comparisons of the number of cells per 100μm of retinal tissue examined.

Western Blot Analysis

For quantitative GFAP/GS protein analysis, the animals were divided randomly into three groups. The first group (n=12) received a single intravitreal injection of BDNF (5μg), the second group (n=12) received only an optic nerve crush, and the third group (n=12) received an optic nerve crush and a single BDNF injection at the time of nerve crush. Each group of animals then was divided into four additional subgroups, corresponding to survival times of 1 day (n=3), 3 days (n=3), 7 days (n=3) or 14 days (n=3). Five rats were used as normal controls, and two additional rats received sham injections consisting of 5μl of sterile saline. No change in either GFAP or GS was observed for these animals. For Western blot analysis, the retinae were dissected in chilled 0.01M PBS, and homogenized in 500μl of 20mM Tris buffer (pH 7.5) using a TissueMite homogenizer (Tekmar, Cincinnati, OH). The buffer contained 1mM sodium orthovanadate (Sigma, St. Louis, MO), 150mM sodium chloride, 10mM sodium fluoride (Sigma), 1mM phenylmethylsulphonyl fluoride (Sigma), 5μg/ml leupeptin (Sigma), 10μg/ml aprotinin (Sigma), 1% NP-40 (Sigma). Samples of the homogenized tissue then were microcentrifuged at 10,000 RPM for 10min at 4°C. Protein concentrations in the homogenized tissue lysate were determined using the Bradford assay kit (Biorad, Hercules, CA). One microgram of protein from each sample was mixed with equal volumes of a buffer containing 30% glycerol, 6% sodium dodecyl sulphate (SDS), 0.075% Bromphenol blue, 15% ß-mercaptoethanol, and 187.5mM Tris (pH7.0). The mixtures then were boiled for 3 min and subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to Immobilon P membrane (Millipore, Bedford, MA) by electroblotting in a solution containing 25mM Tris, 192mM

glycine, 20% v/v methanol, pH8.3. To detect GFAP, the membranes first were incubated with 5% non-fat dry milk in Tris buffered saline (TBS, 20mM Tris, 150mM NaCl, pH 7.5) to block non-specific binding, then treated with a 1:10,000 dilution of a polyclonal anti-GFAP antibody (DAKO), followed by a 1:3,000 dilution of HRP-conjugated rabbit IgG (Santa Cruz Technology). For GS detection, the membranes also were first soaked in a solution of 5% non-fat dry milk in TBS, then incubated with a 1:10,000 dilution of a monoclonal anti-GS antibody (Chemicon), followed by a 1:3,000 dilution of HRP-conjugated horse IgG (Santa Cruz). For both proteins, the immunoblots were washed 3 times (10 min. each) in TBS containing 0.1% Triton X-100 between each step. The blots were visualized by chemiluminescense detection using ECL reagent (Amersham, Arlington Heights, IL) and the protein information then was transferred to Hyperfilm MP (Amersham) for visualization. The films were scanned at 600dpi, and the resulting digital images were analyzed quantitatively using Un-Scan-It gel software (Silk Scientific, Orem, UT). In all cases, protein samples obtained from normal and each group of experimental animals were presented on the same blot so that direct comparisons could be made between normal and experimental animals.

Statistics

The protein analysis data were derived, and are presented, as 'percentage of normal'. They are expressed as Mean ± 1SD. A one sample t-test with Bonferroni's adjustment for multiple comparisons was performed using SPSS software (Chicago, IL), and the level of significance was adjusted to p=0.05/number of comparisons.

RESULTS

Optic Nerve Crush Results in Increased GFAP Expression

Müller cells from normal retinae showed a low level of immunohistochemical staining for GFAP, and this was restricted to the inner endfeet regions of these cells (Fig. 3-1A). The number of GFAP positive profiles had increased significantly by 3 days after the optic nerve crush, with the labeled processes forming parallel arrays that extended from the inner limiting membrane to the outer surface of the retina (Fig. 3-1C). Some processes also formed extensive branches, suggesting lateral interactions among neighboring cells. Not only were GFAP positive processes seen over the entire 2 week post-crush period examined (Figs. 3-1 D & E), but the individual profiles appeared to increase in thickness with longer post-crush durations. As expected, the cell bodies of Müller cells did not show positive staining for GFAP.

Quantitative changes in GFAP expression were assessed from Western blots. To ensure the accuracy of these measurements, however, it first was necessary to establish the linearity of the quantification method. Increasing amounts of total protein (2μg, 4μg, 8μg, 16μg, 32μg, 64μg), obtained from the same retina sample, were run on the gel, detected using the Western blot protocol (Fig. 3-2A), and the signal for each band was quantified using the Un-Scan-It software. Linear regression of the plot comparing protein level with signal intensity produced a r^2 value of 0.98, and a level of significance of $p<0.001$ (Figure 3-2B).

All of the GFAP Western blots displayed distinctive bands with the expected molecular weight of 51kDa. Optic nerve crush resulted in an increase in the intensity of the GFAP signal (Figure 3-3). Quantification of these bands showed that the amount of GFAP protein relative to normal was increased 1.59±0.38-fold at 1 day post-crush, 9.50±5.76-fold at 3 days ($p<0.05$), 7.57±4.62-fold at 7 days ($p<0.05$), and 10.48±5.4-fold ($p<0.05$) at 14 days post optic nerve crush, (Figure 3-4). When BDNF was provided at the time of the crush, the increase in GFAP levels were similar, and not statistically different, from those seen in animals that received optic nerve crush alone. Intravitreal injection of BDNF by itself did not significantly change the level of GFAP protein level (ranges from 0.38 to 1.15-fold above normal, $p>0.05$).

Optic Nerve Crush Results in a Change in the Distribution, but Not Expression of Glutamine Synthetase

In the normal retinae, immunocytochemical staining for GS was heaviest within the Müller cell bodies, although weak label also could be seen among some of the Müller cell endfeet in the ganglion cell layer (Figure 3-5A). At 1 day post-crush the pattern of GS label was basically reversed; now there was very strong GS immunoreactivity within the Müller cell processes located within the ganglion cell layer, but almost no GS within Müller cell bodies (Figure 3-5B). By day 3 post-crush, GS immunoreactivity had reappeared in the cell soma (Figure 3-5C), and 1 week post-crush the pattern of staining was essentially normal (Figure 3-5 D and E). The number of GS positive Müller cell bodies within the INL was counted in the normal and optic nerve crush animals (Figure 3-6). There were 13±1 GS positive cells/100μm in the INL of the normal retinae, but only

7±2 GS positive cells/100μm present 1 day after the nerve crush. The number of GS positive cells per 100um of INL measured at 3, 7, and 14 days post-crush were 8±1, 13±2, and 12±2 cells, respectively. These changes coincided with the shift in GS staining between the cell bodies and the inner processes of Müller cells.

Western blot analysis of the retinae from the normal and optic nerve crush animals showed GS as a single protein band with a molecular weight of about 45kDa. The level of GS protein was not affected by optic nerve crush, intravitreal BDNF injection, or a combination of the two (Figure 3-7 and Figure 3-8).

DISCUSSION

Increased Müller cell GFAP expression has been reported following a wide variety of retinal injuries, including mechanical damage [5], lensectomy-vitrectomy [6], photoreceptor degeneration [7-10], experimental retinal detachment [11,12], diabetic retinopathy [13-15], retinal ischemia [16,17], and experimental [18,19] and human glaucoma [11]. Increased levels of GFAP in Müller cells also has been observed following optic nerve transection [20,21]. Taken together, these data have lead to the general acceptance that increased expression of GFAP by Müller cells is a reliable indicator of retinal neuropathy. In the present study we show that optic nerve crush also results in an increase in GFAP expression by Müller cells. We found that GFAP levels increased ~7-9 fold relative to normal within 3 days of the optic nerve damage, and that they remained at this elevated level over the entire 2 week period examined. Although BDNF has a survival promoting effect on RGCs (Chapters I and II), when applied alone, it did not appear to affect GFAP expression by Müller cells.

Although the immunohistochemical analysis identified Müller cells as the primary source of increased retinal GFAP following optic nerve crush (Figure 3-1), it is important to note that they are not the only source of GFAP in the retina. Previous studies have shown that there is a population of astrocytes within the nerve fiber layer that also produce GFAP. Although these cells are not easily distinguishable from the endfeet of Müller cells when viewed using retinal cross-sections, they are clearly visible on retina whole mounts. It is unlikely, however, that these glia contributed to the increase in GFAP levels seen here, since it has been shown that these retinal astrocytes do not increase their levels of GFAP expression in response to optic nerve transection [20,21] This lack of a response to nerve injury might result from the fact that these retinal astrocytes appear to be most closely associated with the retinal vasculature, and not ganglion cell axons [20,21].

The increase in GFAP expression by Muller cells coincides with the onset of RGC degeneration, but takes place before any significant level of RGC death has occurred (day 5) [22]. This suggests that Müller cells are sensitive to early degenerative changes within the retina that might not necessarily involve actual ganglion cell death. Although the stimuli that induce increased GFAP expression by Müller cells are unknown, several possible candidates exist. One possible candidate is reduced neuronal electrical activity. Reduced afferent input has been shown to result in increased GFAP expression at the frog neuromuscular junction [23], within the chick cochlear nucleus [24,25], and in the rat lateral geniculate nucleus [26]. Optic nerve crush has been shown to reduce RGC activity [27]. Since Müller cell processes are intimately associated with synapses within the retina plexiform layers, and since they are capable of detecting changes in neuronal activities via neurotransmitter receptors [28,29], it is plausible that a reduction in

ganglion cell activity also may signal the Müller cells to increase their levels of GFAP expression. Long-term increase in GFAP level, however, most likely is due to soluble factors released by neurons and glia. In particular, transforming growth factor, fiberoblast growth factor, and cilliary neurotrophic factor (CNTF) [30], have been shown to be present at increased levels following neuronal injury.

The fact that BDNF did not affect GFAP expression in the normal retina was not unexpected. BDNF also has been shown to have no affect on GFAP expression in cultured fetal rat astrocytes [31]. Similarly, in their study of the ability of BDNF to rescue photoreceptors from degeneration, Lewis et al. [32] found no change in GFAP expression following injection of BDNF into the normal eye. This result suggests that GFAP expression within the retina is not directly linked to the signaling cascades of BDNF.

We did, however, expect BDNF to reduce GFAP expression following optic nerve injury. This expectation was based on our previous demonstration that BDNF promotes RGC survival and reduces neuronal degeneration following optic nerve injury (Chapters I and II). In addition, Lewis et al found that BDNF attenuated GFAP expression following retinal detachment [32]. In contrast, we found that application of BDNF at the time of the nerve crush produces changes in GFAP expression that are not different from those measured following optic nerve crush alone. These differences, however, might be explained by several factors, including the animal species used (rats vs cats), model of injury (optic nerve crush vs retinal detachment), time period examined (1-14 days, 4 time points vs 28 days, one time point), and GFAP quantification methods (western blot vs image analysis).

Although the overall level of GFAP was not affected by BDNF, there is evidence that BDNF may affect the turnover rate of GFAP. In a pilot study I noticed the presence of multiple bands, rather than a single band, on each GFAP western blot (see Figure 3-3). Adjusting the concentration of ß-Mercaptoethanol in the gel-loading buffer reduced the intensity of the lower band, but did not necessarily eliminate it. While the lower band could be seen under most conditions, it was particularly prominent in the crush + BDNF animals. One possible explanation for the multiple bands is post-translational modification of GFAP. Since phosphorylation or glycosylation results in a slight increase in protein molecular weight, it is possible that the upper bands reflect phosphorylated/glycosylated GFAP. Similarly, because stepwise proteolytic breakdown of GFAP has been shown to produce multiple bands with lower molecular weights [33], it is possible that the lower bands represent the result of this process. Although Western blot analysis does not allow precise determination of molecular weight, comparing the positions of the different bands with the normal data suggests that the lower bands in our gels most likely are the result of proteolytic cleavage of the 51kDa GFAP protein (upper band). Since the overall GFAP content in the crush + BDNF group was not statistically significant from that of the crush only group (upper bands of each), the very prominent lower band (degraded GFAP) for the BDNF + GFAP animals suggests that in actuality more BDNF was produced in these animals.

Since its discover in the 1970's, the function of GFAP has remained a mystery [34]. Recent advances in genetics have made it possible to generate mice that carry a defective GFAP gene, and thus are incapable of expressing GFAP. At present, there are at least four different lines of GFAP-null mice [35-38]. To the surprise of many, these mice undergo

normal development and achieve adulthood without showing any anatomical, histological or behavioral abnormalities. In addition, the astrocytes in these mutant mice continue to respond to injury, although quantification of the response is difficult without GFAP as a marker [36]. The absence of an obvious phenotype does not appear to be due to a compensatory up-regulation of other glial intermediate filaments, such as vimentin or nestin [35-37]. Subsequent studies, however, have demonstrated enhanced long-term potentiation in the hippocampus [37] and deficient long-term depression in cerebellar Purkinje cells [39] of GFAP-null mice, which supports the idea that GFAP plays a role in regulating synaptic transmission, although its mechanism remains a mystery. In general, the increased GFAP expression by retinal Müller cells in response to optic nerve injury represents a sign of Müller cell plasticity in response to a changing neuronal environment.

One of the more interesting findings of these studies was the change in the pattern of GS labeling that occurred as a result of the optic nerve injury. Because the shift from dense labeling within the Müller cell somata to the inner retina did not involve an increase in GS protein levels, it seems reasonable to assume that the initial response of Müller cells to optic nerve injury involves a rapid translocation of GS from the cell soma to their inner endfeet near the initial site of ganglion cell injury. This response is not surprising, however, since Müller cells play a primary role in the control of retinal glutamate, and glutamate has been shown to be excitotoxic in the retina, as well as in other areas of the CNS; direct application of glutamate to the retina results in retinal ganglion cell death [3], and the degenerative effects can be alleviated by the application of glutamate receptor antagonists [40].

Unlike GFAP, we found that the level of glutamine synthetase in the retina was not affected by BDNF application, optic nerve damage, or a combination of both. This lack of change in overall GS expression might be due to the fact that photoreceptors, and not ganglion cells, are a major source of glutamate within the retina. Thus, the small additional amount of glutamate released by degenerating RGCs may not increase overall glutamate levels enough to induce a change in GS production. Increasing the availability of GS, however, has been shown to be beneficial for the survival of RGCs. Vardimon [41] reported that application of purified GS into retinal explants promoted the survival of RGCs. Since our work, and that of others, has shown that that BDNF can reduce RGC death following optic nerve injury, without increasing GS expression, it will be interesting to know whether a combination of BDNF and GS might result in even greater ganglion cell survival than either treatment alone. That GS levels did not increase in a manner similar to those seen for GFAP is not surprising, as Mizutani et al. [14] found a similar dissociation between GS and GFAP expression in human diabetic retinopathy

In summary, this set of experiments has demonstrated that RGC death induced by optic nerve crush is accompanied by changes in Müller cells. They increase expression of GFAP and shift GS to the RGC layer, suggesting a protective role in preventing RGC degeneration. Despite the fact that BDNF promoted the survival of RGCs (Chapters I and II), it did not affect the retinal response of either GFAP or GS. These results suggest that BDNF act directly on RGCs, although this set of experiment cannot exclude the possibility that BDNF acts through other molecules expressed by Müller cells.

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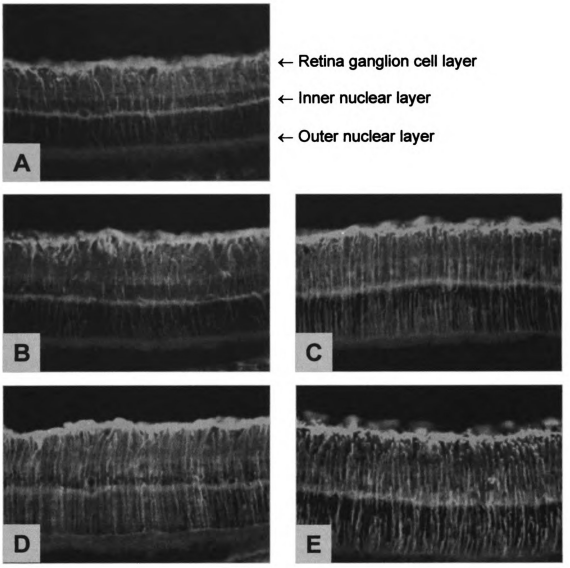

Figure 3-1. Immunofluorescent staining for GFAP revealed limited staining in the inner processes of Müller cells in normal retina (A). Little has changed in this expression pattern 1 day after optic nerve crush (B). However, the level of GFAP expression is significantly greater than normal 3 days post nerve crush (C), and now also includes Müller cell outer processes. High level of GFAP expression was maintained through 7 days (D) to 14 days (E) post optic nerve crush. At on time was the cell bodies of Müller cells positive for GFAP.

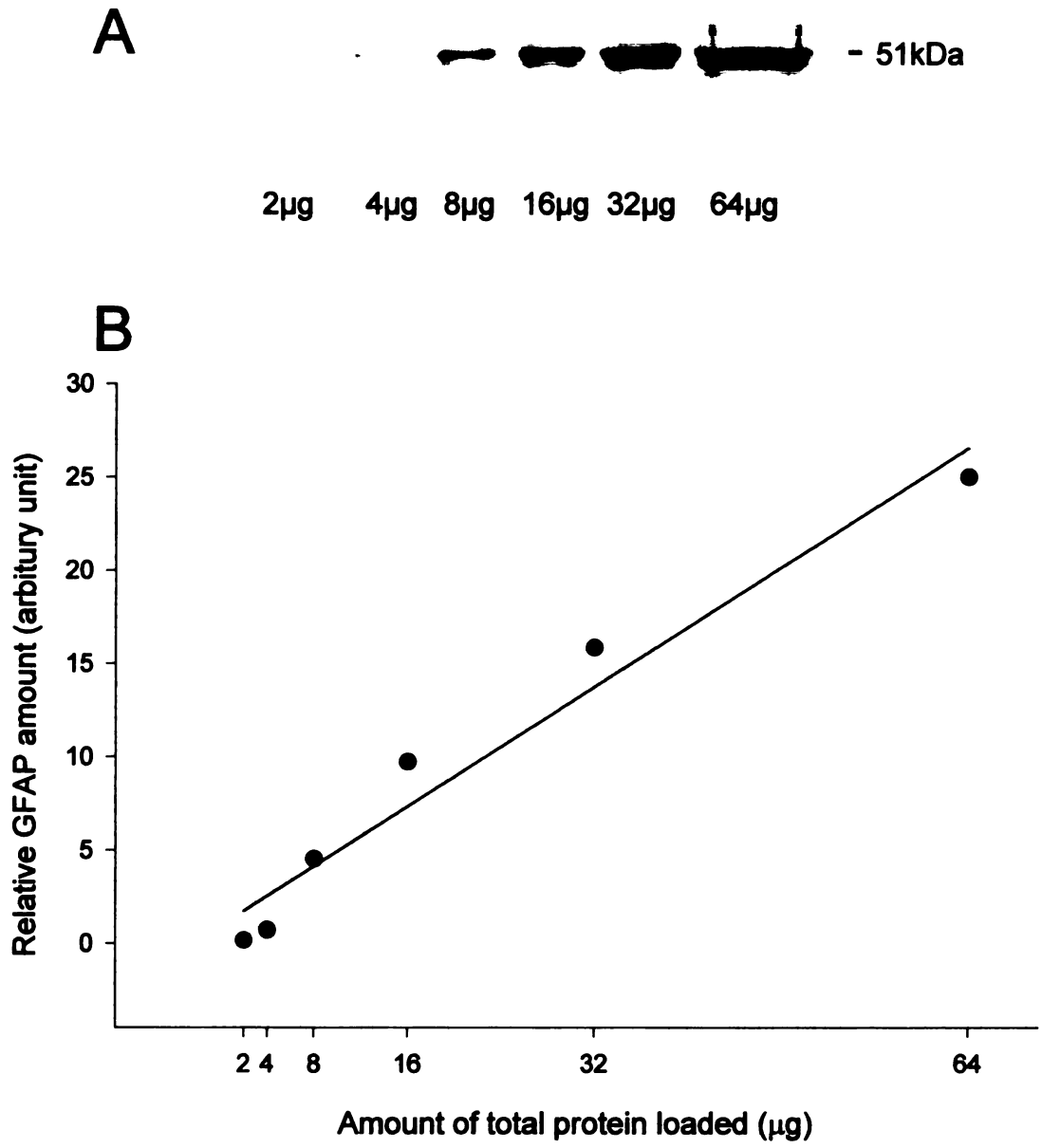

Figure 3-2. The linearity of GFAP Western blot was tested by loading increasing amounts of protein from the same retina sample into parallel lanes. GFAP was visualized (A) and quantified. Linear regression of protein amount vs GFAP signal intensity showed a r^2=0.98 and p<0.001(B).

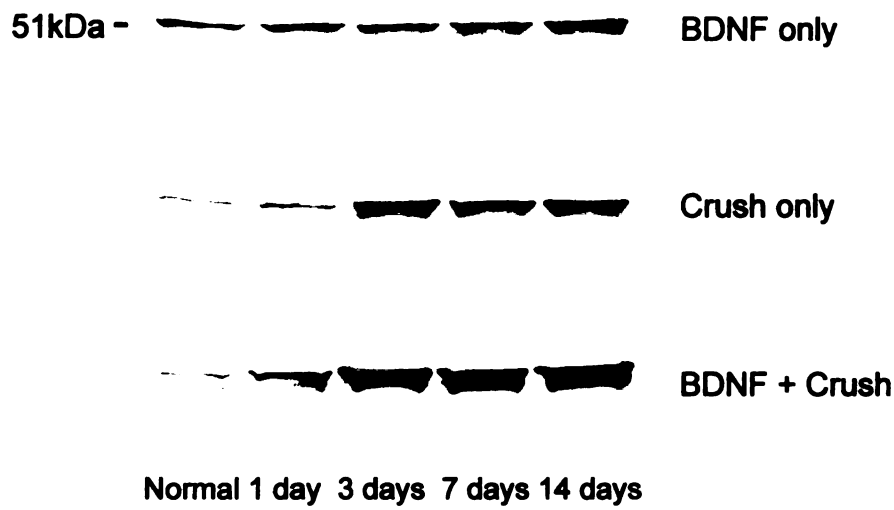

Figure 3-3. Glia fibrillary acidic protein (GFAP) was detected using Western Blot. Rat retinae were dissected and homogenized in protease inhibitors cocktail. Equal amounts of total retina protein were loaded for the samples. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. GFAP was visualized by using specific antibodies and ECL chemiluminescence detection kit. The molecular weight of the detected bands is approximately 51kDa. Top panel: BDNF application only group; Middle panel: optic nerve crush only group; Bottom panel: optic nerve crush and BDNF application group.

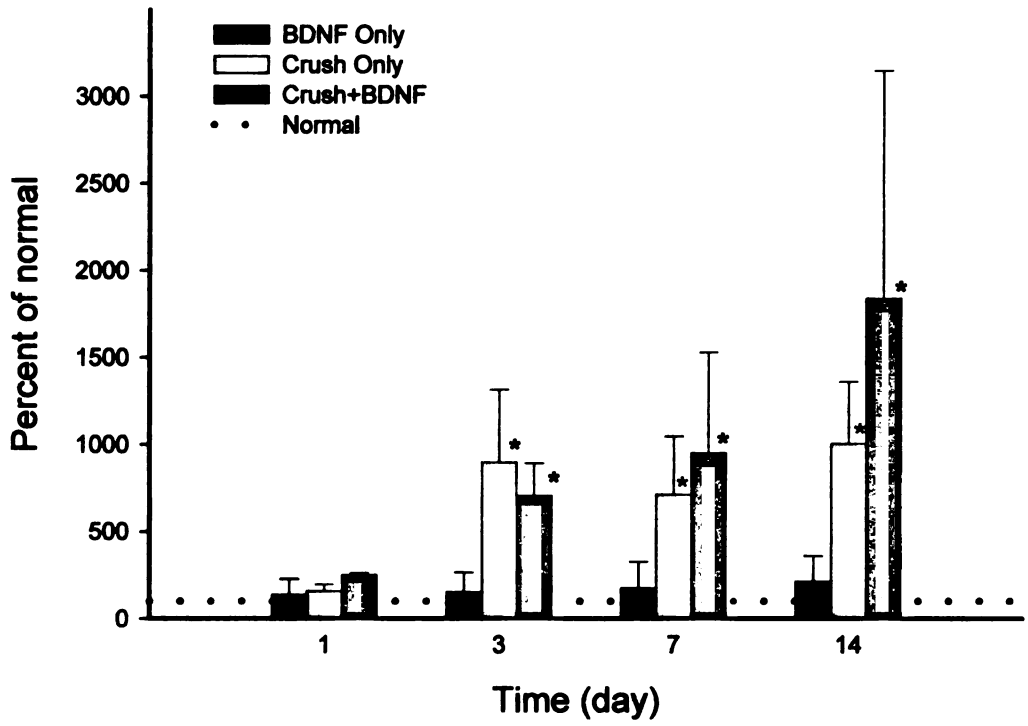

Figure 3-4. Semi-quantitative analysis of the GFAP protein showed that BDNF injection alone caused only a slight increase in GFAP protein levels ($p>0.05$), while optic nerve crush and the combined crush/BDNF application treatment caused a significant increase in GFAP protein compared with normal, but the difference between the two was not significant statistically. * $p<0.05$, compared to normal. Error bars are ± SD.

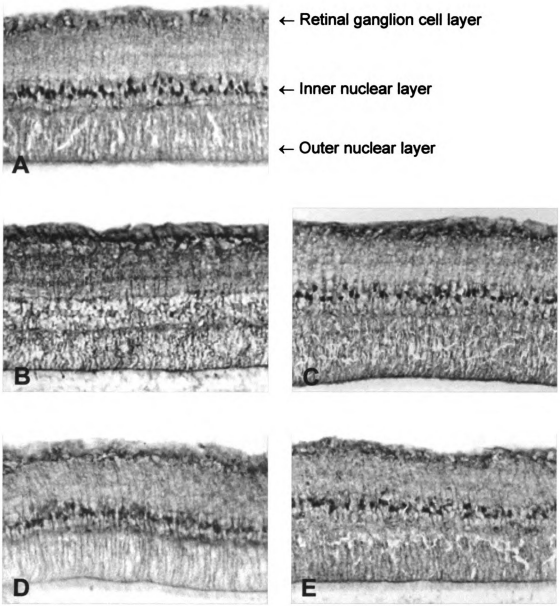

Figure 3-5. Immunohistochemistry staining for GS showed that positive staining is seen in association with Müller cell processes and their cell bodies. In normal retinae (A), GS immunoreactivity is strongest within the cell soma, while at one day post-crush (B) the highest level of label appears within the inner processes, near the ganglion cell layer. The immunoreactivity was shifting back toward the INL 3 days (C) and by day 7 (D) and day 14 (E) the pattern of staining has returned to normal.

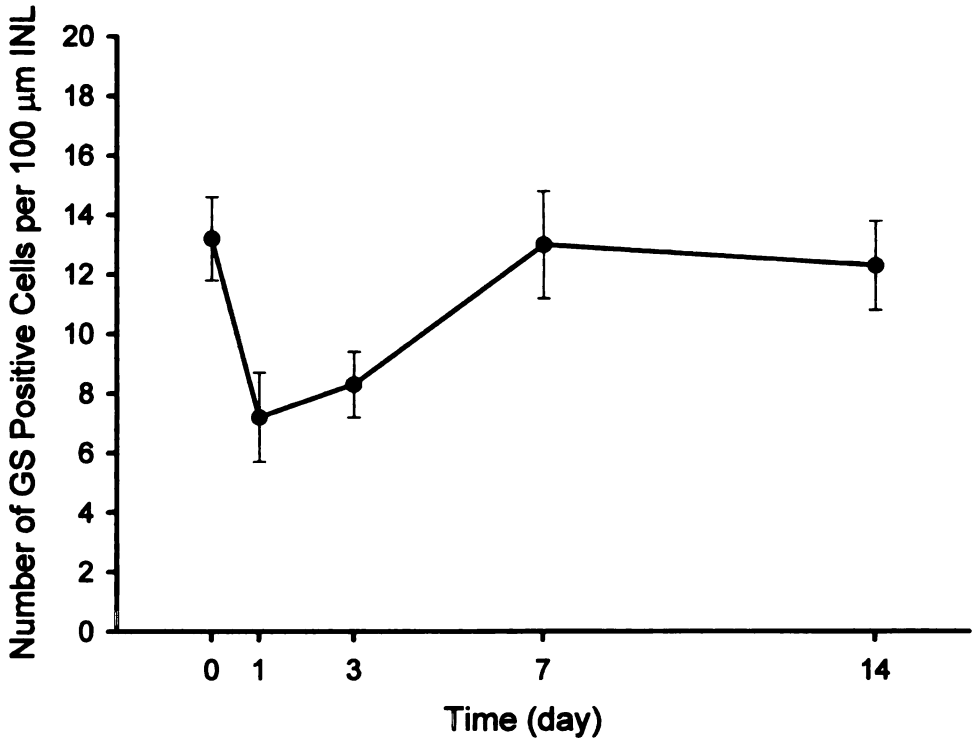

Figure 3-6. Number of GS positive Müller cells within the inner nuclear layer decreased at day 1 post-crush. This coincided with the shift in GS staining from the cell body to the inner processes as seen in Figure 3-5. Error bars are ± SD.

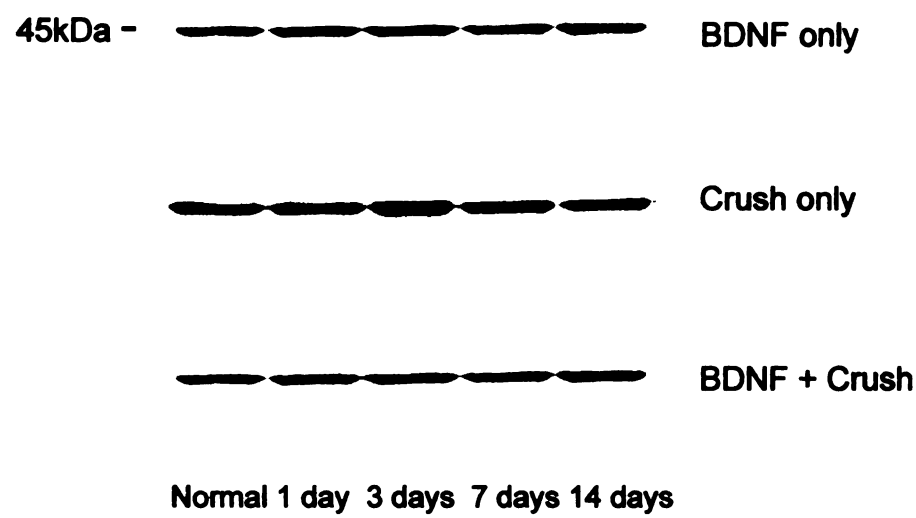

Figure 3-7. Glutamine synthetase (GS) was detected using Western Blot. Rat retinae were dissected and homogenized in protease inhibitors cocktail. Equal amounts of total retina protein were loaded for each sample. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. GS was visualized by using specific antibodies and ECL chemiluminescence detection kit. The molecular weight of the detected bands are approximately 45kDa. Top panel: BDNF application only group; Middle panel: optic nerve crush only group; Bottom panel: optic nerve crush and BDNF application group.

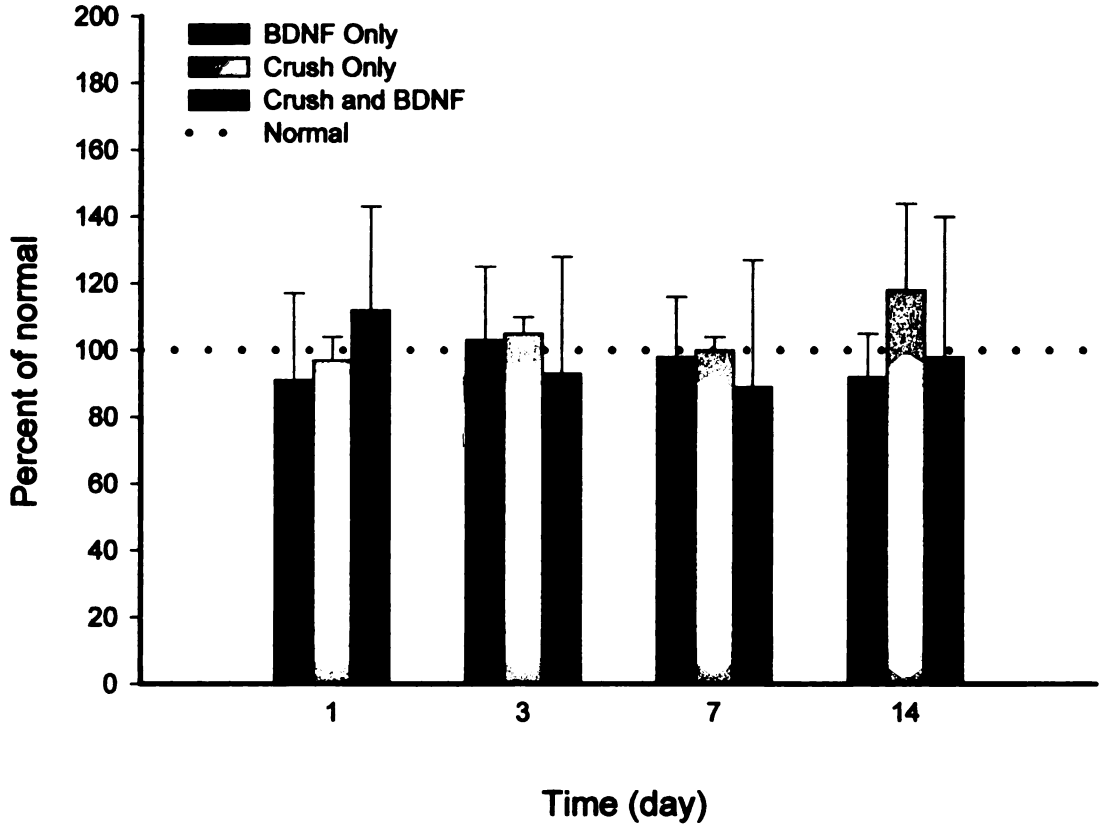

Figure 3-8. Semi-quantitative analysis of glutamine synthetase level showed that the amount of glutamine synthetase protein in the retinae of rats was not affected by either BDNF application, optic nerve crush, nor a combination of the two procedures. Error bars are ± SD.

Chapter IV

Brain-Derived Neurotrophic Factor Reduces TrkB Protein and mRNA in the Normal Retina and Following Optic Nerve Crush in Adult Rats

INTRODUCTION

Because many ocular diseases involve retinal ganglion cell death, and because the retina is an approachable part of the brain, numerous studies have used the retina as a model to study degeneration and neuroprotection within the CNS. Of the different pharmacological approaches being studied, direct application of neurotrophins to the injured nervous system has produced some of the most promising results. Indeed, the ability of BDNF, a member of the neurotrophin family, to enhance retinal ganglion cell survival following optic nerve damage has been well characterized in the rat [1-9]. Previously, we demonstrated that BDNF also is effective in preventing RGC death following optic nerve crush in the cat (Chapter II), which has eyes that are comparable in size and volume with those of primates. While this suggests that BDNF might be a reasonable therapeutic for use in humans, the data also indicated some limitations of the drug. Namely, that increasing the dose above 30μg BDNF resulted in the survival of fewer, rather than more, ganglion cells, and that multiple injections did not have an additive effect on ganglion cell survival. The purpose of this set of experiments was to investigate one possible mechanism behind the self-limiting effects of BDNF.

The biological effects of BDNF are mediated primarily by its high affinity receptor, the tyrosine receptor kinase B (This chapter follows the convention of using 'TrkB' to represent the protein and 'trkB' to represent the mRNA). This receptor exists in full-length (TrkB-FL) and truncated forms [10]. TrkB-FL is a 145kDa protein that contains intracellular tyrosine kinase domains that become phosphorylated upon BDNF binding. This phosphorylation then initiates an intracellular signaling cascade. The truncated TrkB receptor is a 95kDa protein. It lacks the tyrosine kinase domain, and therefore is not able to initiate a signaling cascade. Immunohistochemistry and *in situ* hybridization studies have shown that TrkB is expressed by cells of the developing and adult mammalian retina, and especially by cells in the RGC and inner nuclear layers [11-15].

While much work has focused on the ability of BDNF to promote ganglion cell survival in the injured retina, little is known about the underlying mechanisms. Of particular importance is the effect that BDNF has on the regulation of the TrkB receptor. The experiments presented in this Chapter show that injections of BDNF into the normal eye result in a rapid and significant reduction in TrkB-FL and trkB-FL content, but they have no effect on the truncated form of TrkB. A similar decrease in TrkB-FL protein and mRNA also was observed following optic nerve crush, with or without BDNF treatment.

MATERIALS AND METHODS

Animals and Surgical Procedures

Adult Sprague-Dawley rats weighing 250-300g were obtained and maintained as described in Chapter 1. Surgical procedures concerning the optic nerve crush and BDNF injection also were the same as those described previously.

Western Blot Analysis for TrkB

For quantitative TrkB analysis, the animals were divided randomly into three groups. The first group (n=12) received a single intravitreal injection of BDNF (5μg), the second group (n=12) received only an optic nerve crush, and the third group (n=12) received an optic nerve crush as well as a single BDNF (5μg) injection at the time of the nerve crush. Each group of animals then was divided into four additional subgroups, corresponding to survival times of 1 day (n=3), 3 days (n=3), 7 days (n=3) or 14 days (n=3). Three additional rats were used as normal controls, and two rats received sham injections consisting of 5μl of sterile saline. The surgeries were scheduled so that all animals were sacrificed on the same date. This reduced variations caused by sample processing and tissue storage.

The TrkB Western blot protocol used in these studies was a modified version of that used by Frank et al [16]. In brief, retinae were dissected and homogenized as described previously (Chapter III) for GFAP/GS Western blot analysis, and the protein concentration of each sample was determined. One microgram of protein from each sample then was added to 200μl of beaded agarose wheat germ lectin (Amersham Pharmacia, Piscataway, NJ) and the mixture was incubated overnight at 4°C to precipitate all glycosylated proteins, including TrkB. The precipitates then were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. The separated proteins were electroblotted to Immobilon P membrane (Millipore, Bedford, MA) at 4°C in 10mM 3-cyclohexylamino-1-propanesulphonic acid buffer (pH11) containing 10% methanol. To detect TrkB, the membranes were first

incubated in CAS blocker (1 hr @ room temperature: RT, Zymed), followed by incubation in a 1:2,000 dilution of a monoclonal anti-TrkB antibody (overnight, 4°C, Transduction labs, Lexington, KY). They then were incubated in a 1:20,000 dilution of HRP-conjugated mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Between each step, the immunoblots were washed 3 times (10 min each) in TBS containing 0.1% Triton X-100. A SuperSignal West Femto kit (Pierce, Rockford, IL) was used for final protein visualization. The films were developed and analyzed as described in Chapter III. In all cases, one homogenated sample from an eye representing each survival period and from a normal animal were placed on the same blot so that direct comparisons could be made between normal and experimental animals.

RNase Protection Assay (RPA) for Full-length trkB

The animals used for analyzing trkB mRNA received the same treatments as those for TrkB protein analysis, except that 3 animals were added to the BDNF only with 14 day survival period group. RPA was selected as the method to quantify the amount of full length trkB in the retinal tissue because of its sensitivity and reliability. This assay utilizes labeled, sequence specific, RNA as a probe that selectively hybridizes with the desired target mRNA in the sample (here, trkB). The resulting double stranded RNA is selectively protected from RNase, an enzyme that digests only single stranded RNA, while unwanted RNAs and unhybridized probe are digested by the enzyme. RNase digestion is followed by gel electrophoresis and electroblotting of the protected RNA fragment to a nylon membrane. The mRNA of interest then is detected according to the labeling method (e.g. radioisotopes, Biotin, etc.) used to generate the RNA probes.

To prepare a biotin-labeled RNA probe specific for the full-length TrkB receptor, its sequence (accession number M55291) first was retrieved from GenBank (http://www.ncbi.nlm.nih.gov). Because the sequence from nucleotide 2019 to 2203 corresponds to a portion of the intracellular domain of the full-length TrkB receptor that is not present in the truncated TrkB receptor, this sequence was chosen as the template for generating the full-length trkB probe. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify the chosen sequence of nucleotides so that it could be used as a DNA template to generate the RNA probe. Netprimer software (PrimierBiosoft, Palo Alto, CA) was used to assist with the primer selection. The forward primer used was 5'-attaaccctcactaaagggagaTGCTCAAGTTGGCGAGACA-3' and the backward primer was 5'-taatacgactcactataGGGGTTTTCAATGACAGGGA-3'. Note that the T3 and T7 bacterial phage RNA promoter (identified in lower case) was incorporated into the forward and backward primers, respectively. This was done to allow direct *in vitro* transcription of the RT-PCR products [17]. The primers were synthesized by the Macromolecular facility at MSU. Total RNA was isolated from normal rat cerebellum using TriZol reagent (Lifetech, Grand Island, NY), and the RT-PCR was performed using a one-step kit (Qiagen, Valencia, CA). The product was run on 1.5% agarose gel and the band around 200bp was excised and purified using the QiaEX II gel extraction system (Qiagen). The nucleotide sequence of this purified PCR product was obtained by automated fluorescent DNA sequencing (MSU sequencing lab), and was verified to be identical with the selected fragment. The sequence-identified PCR product then was used as a template to synthesize biotin-labeled RNA using a Maxiscript kit (Ambion, Austin, TX) with T7 as the promoter. Biotin-14 CTP (BRL Gibco, Grand Island, NY) was

incorporated into the reaction at the ratio of 4:6 relative to unlabeled CTP. The synthesized RNA then was gel purified and used as the probe for full-length trkB.

To quantify trkB, retinae were dissected in chilled 0.01 M PBS and stored at minus 70°C until processed (< 2 weeks). Total RNA was isolated from the retina tissue using Trizol reagent (Lifetech), and the RNase protection assay was performed using an RPA III kit (Ambion). In brief, biotin-labeled trkB probe (300pg), biotin-labeled rat cyclophilin probe (300pg, synthesized from a plasmid purchased from Ambion) and total retina RNA (10-20μg) were co-precipitated with ethanol, denatured at 96°C for 3min, and hybridyzed overnight at 42°C. RNA digestion was carried out at 30°C for 30min. Subsequently, the protected fragments were precipitated and dissolved in 7μl of gel loading buffer. The samples then were loaded onto a 5% acrylamide urea gel and electrophoresed at constant current (25mA) for 90min in Protean II xi cell (Biorad). A RNA size marker (Ambion) was loaded into the first well of each gel. RNAs then were electroblotted to positively charged nylon membranes (Ambion) using a semi-dry transfer cell (Biorad) with a constant current of 0.3mA/cm^2 of membrane area. The membranes then were baked at 80°C for 15min to cross-link the RNA to the membrane. The biotin signal was detected using the BrightStar biotin detection kit (Ambion). In brief, the membranes were washed and incubated in a streptavidin-alkaline phosphatase conjugate (30min @RT), followed by incubation in a chemiluminescence reagent (5min @RT). The signal on the membrane was transferred to Hyperfilm (Amersham) using a 0.5-1 hour exposure period, and the film was developed in GBX developer (Kodak, Rochester, NY). The images were scanned at 600dpi and analyzed using Un-Scan-It gel software (Silk

Scientific)

Statistics

The protein and mRNA data were derived, and are presented, as 'percent of normal'. All values are expressed as Mean ± 1SD. A one sample t-test with Bonferroni's adjustment for multiple comparisons was performed using SPSS software (Chicago, IL), and the level of significance was adjusted to p=0.05/number of comparisons.

RESULTS

Western Blot and RNase Protection Assay for TrkB in Normal Rats

The monoclonal antibody used to obtain the TrkB Western blots is specific for the extracellular domain of TrkB, and thus should detect both full-length and truncated TrkB receptors. As expected, two distinct bands with molecular weights of 145kDa and 95kDa (Figure 4-1) were detected on each blot, and the specificity of the antibody was confirmed using cerebellar tissue as a positive control. Several bands of lower molecular weight also were visible in the retinal samples, but this most likely was due to the extended exposure time (10-15min) needed to detect the low amount of TrkB present in a single rat retina. Similar bands were not seen with respect to the cerebellar tissue (<2min exposure). The RNase protection assay (Figure 4-2) showed two clear bands for each lane of sample. These corresponded to the expected sizes of the protected fragment of the trkB (185bp) and cyclophilin (103bp) probes. The sham injections did not cause any changes in TrkB or mRNA levels, as detected by Western blot or RPA analysis (data not shown).

Effects of BDNF on TrkB/trkB protein and mRNA in Normal Adult Rat Retina

The amount of full-length and truncated TrkB in the retina was examined 1, 3, 7, and 14 days following a single intravitreal injection of BDNF (5ug) As shown in Figure 4-3, the level of full-length TrkB was reduced to only 4.8±2.07% of normal 1 day following the BDNF injection. Retinal levels of full-length TrkB recovered slowly, reaching 17.7±13.73% of normal at day3, 13.4±2.0% at day 7, and 31.4±8% at day14 ($p<0.01$ for each group).

One day after the BDNF injection into the normal eye, trkB mRNA was reduced to 42.2±11.14% of normal ($p<0.05$). The level of trkB then increased rapidly, reaching 147.1±27.92% of normal by day 3, then falling to 94.6±4.40% of normal at day 7 and 48.8±9.35% ($p<0.01$) by day 14 (Figure 4-3).

Effects of Optic Nerve Injury on Retina TrkB/trkB Protein and mRNA

Effects of optic nerve injury on retinal levels of TrkB/trkB were examined 1, 3, 7, and 14 days post-crush. As shown in Figure 4-4, full-length TrkB remained at normal levels for about 7 days (day 1: 112.7±34.39%; day 3: 93.7±31.01%; day7: 96.7±64.47%) then decreased to 54.0±22.91% at day 14 post-crush ($p>0.05$ for each group).

Optic nerve crush did not initially result in a significant change in the level of trkB mRNA (day 1: 77.5±11.76% of normal level; $p>0.05$). By day 3 the level had increased to 159.3±3.12% of normal ($p<0.01$), and it then returned to a level that was not statistically different from normal by day 7 (87.9±47.29% of normal, $p>0.05$). By day 14

post optic nerve crush, however, the level of trkB-FL mRNA was significantly lower than normal (40.2±11.74%, $p<0.05$) (Figure 4-4).

Effect of BDNF on Retina TrkB/trkB protein and mRNA after Optic Nerve Crush

The combined effect of optic nerve crush and intravitreal administration of BDNF on TrkB/trkB also was examined (Figure 4-5). The levels of full-length TrkB present at days 1, 3, and 7 post-crush + injection were 13.8±7.5%, 8.4±2.50%, and 12.7±12.88% , respectively ($p<0.05$ for each group). Full-length TrkB had recovered to about 61.7±27.06% of normal at day 14 ($p>0.05$).

A significant decrease in trkB also was seen in response to the ON crush + BDNF injection. At 1 day post crush + injection, mRNA levels were only 21.9±3.60% of normal ($p<0.01$). They increased to 65.1±25.05% and 90.7±29.74% of normal by day 3 and day 7, respectively. By day 14, however, the level of trkB again was only 29.75±5.49% of normal ($p<0.01$; Figure 4-5).

The level of truncated TrkB protein did not change significantly over any of the time points examined (Figure 4-6). However, when BDNF was applied to optic nerve crushed animals, it reduced truncated TrkB to ~50-60% of normal between day 1 and day 3. This initial reduction was recovered to ~81% of normal by day 7 and to 110% of normal by day 14.

DISCUSSION

The data presented here show that: 1) intravitreal application of BDNF alone produces a rapid, dramatic, and long lasting reduction in the level of full length TrkB

receptor protein (TrkB-FL) in the normal retina, 2) TrkB-FL also is reduced following optic nerve crush, but this reduction is not evident until 14 days post-crush, when approximately 80% of the RGCs have died, and 3) combining BDNF treatment with ON injury results in a pattern of TrkB-FL reduction that is similar to that seen following BDNF treatment alone. In contrast, none of these experimental paradigms appeared to have a significant affect on the amount of truncated TrkB receptor protein within the retina.

Although changes in TrkB-FL were accompanied by changes in trkB mRNA expression, the changes in receptor protein and mRNA did not always coincide with one another. This was not too surprising, however, since protein production is influenced by many factors, and mRNA is only one such factor. In fact, the inability to predict mRNA levels based on protein expression has been highlighted recently by Gygi et al.[18], who examined over 150 protein-mRNA pairs.

While the ability of BDNF to promote RGC survival following optic nerve injury is well-known [1-9], it is clear from our studies, and those of others, that higher doses, multiple injections, and chronic application of the drug do not necessarily translate into enhanced levels of neuroprotection (see Chapter II, and ref. [2,6]). One possible explanation for this is that BDNF has a self-regulating effect on its own receptor. This possibility is supported by the data presented here, where we show that BDNF alone results in approximately a 95% reduction in TrkB-FL within 24 hrs of treatment. Thus, BDNF appears to self-limit its neuroprotective ability by down regulating the high affinity receptor it uses to activate intracellular signaling pathways. Down-regulation of TrkB-FL is consistent with other studies showing that BDNF has a similar effect on TrkB-FL in

cultured neurons [19-21] and midbrain neurons in vivo [16]. Although the mechanism underlying BDNF-induced TrkB down-regulation remains unknown, the degradation of other receptor tyrosine kinases, such as the platelet-derived growth factor beta-receptor [22,23] and epidermal growth factor receptor [24], has been shown to involve a small protein (76 amino acids) called ubiquitin. Ubiquitin can be linked covalently to other proteins, and to itself. The attachment of ubiquitin (ubiquitination) can therefore lead to the formation of polyubiquitin chains, which tag the ubiquitinated protein for degradation by the 26S proteasome. Based on these data, a similar ubiquitin-proteosome mechanism has been suggested for TrkB degradation [21].

A second mechanism that might underlie the decrease in TrkB-FL following BDNF injection alone is a reduction in trkB-FL mRNA, however, studies in this area have produced mixed results. While a BDNF-induced down regulation of full length receptor mRNA has been reported in cultured neurons [19,20], in vivo studies by Frank et al. [16], found no change in trkB-FL following 12 days of continuous infusion of the drug into the olfactory bulb. In the present study, we found both an increase and decrease in trkB-FL over the 2 week period studied (Figure 4-3). The difference between our results and those of Frank et al., might be explained by differences in experimental design. Frank et al. used a continuous drug infusion paradigm and examined trkB-FL levels at only one time point (12days post-treatment). We used a single drug application and measured changes in trkB-FL expression over a two-week period. In this regard, it is of interest to note that although BDNF produced a ~58% reduction ($p<0.01$) in the level of trkB-FL in our 1 day and 14 day animals, the levels measured on days 3 and 7 were not significantly different from normal. Thus, it is possible that Frank et al., might also have found a

change in trkB-FL had they examined survival periods that were shorter/longer than the 12day period studied.

While the higher than normal levels of trkB-FL measured 3 days after BDNF injection in normal eyes might reflect synthesis of new mRNA to compensate for the increased degradation of receptor protein (TrkB-FL), the lower than normal levels seen 2 weeks post-injection are more difficult to understand. One possible explanation is that this decrease reflects a problem with the mRNA analysis technique. It is conceivable that an error in the level of cyclophilin, which was used as an internal control to monitor the amount of retina sample loaded onto the gel, could have contaminated the results. This seems unlikely, however, since re-computation of the data using total RNA as the external control produced a similar result (data not shown). Similarly, the addition of a second set of normal animals with these experimental conditions yielded results that were similar to the original measurements. One could speculate, however, that the temporal differences in trkB-FL simply reflect differential expression of this mRNA by the different classes of cells within the retina.

Optic nerve crush by itself resulted in changes in trkB-FL that were comparable to those seen following BDNF injections into the normal eye (c.f. mRNA data for Figures 4-3 and 4-4). Previous studies have shown that the effects of CNS injury on trkB-FL can vary widely, depending on the type and severity of the injury [25,26,26-28]. Mild damage generally results in an increase in trkB-FL, perhaps as a protective mechanism, while more severe injuries result in a decrease in mRNA, most likely due to a loss of the cellular machinery used in mRNA synthesis. While the initial decrease in trkB-FL is difficult to understand, the increased levels 3 days after the optic nerve crush might

reflect an initial response to the nerve injury and the decrease in trkB expression seen at 14 days post-crush most likely results from the significant loss of RGCs, the major source of retinal trkB-FL, that has occurred by this time period [11,14]. RGC death might also mask the full effect seen with respect to changes in TrkB-FL protein in these animals. When one considers that ~30% of the RGCs are lost by day 7, the apparent lack of change in TrkB-FL might actually reflect an increase in TrkB-FL production by the surviving RGCs. However, it also is possible that the increase in TrkB-FL is of non-ganglion cell origin.

When BDNF was administered at the time of the optic nerve crush, there still was a significant decrease in the expression of both TrkB-FL and trkB-FL. This reduction most likely underlies the unresponsiveness of RGCs to subsequent administrations of BDNF, as seen in our cat work (Chapter II) and other studies [2,6]. One interesting observation from this particular group of animals is that the level of full length protein measured 14 days following an optic nerve crush and BDNF injection is twice that measured following the BDNF injection alone (62% vs 31%; c.f. Figures 4-3 and 4-5) This again suggests that either surviving RGCs are producing higher than normal levels of TrkB-FL, that non-ganglion cells have increased their expression, or both.

Most of the details concerning the signaling cascade that occurs after BDNF binds to the TrkB-FL receptor have only recently been described. The binding of BDNF to the full-length receptor first brings multiple TrkB molecules in proximity of each other, allowing transphosphorylation of their intracellular domains to occur. The ligand-receptor complex then is internalized [29]. This leads to a rapid phosphorylation of the trancription factor cAMP response element-binding protein (CREB) and the expression of Fos protein

[19,29]. The activated TrkB receptor also initiates several intracellular signaling pathways, including the Ras/Raf/MAP kinase pathway [30,31] and the phosphatidyl-inositol-3'-kinase (PI-3K) pathway [31,32]. Changes of these diverse signaling pathways in normal RGCs, and after BDNF application require further investigation in order to better understand the complex responses of ganglion cells to injury and different neuroprotection strategies.

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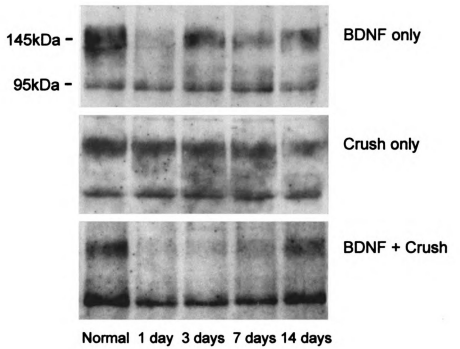

Figure 4-1. TrkB protein was detected using Western Blot. Rat retinae were dissected and homogenized in protease inhibitors cocktail. Equal amounts of total retina protein were mixed with wheat germ lectin-agarose beads to precipitate glycosylated proteins, including TrkB. Precipitated proteins were separated by electrophoresis of SDS-polycrylamide gel and transferred to PVDF membrane. TrkB was visualized by using specific antibodies and chemiluminescence detection reagents. The molecular weight of the detected bands are approximately 145kDa and 95kDa. Top panel: BDNF application only group; Middle panel: optic nerve crush only group; Bottom panel: optic nerve crush and BDNF application group.

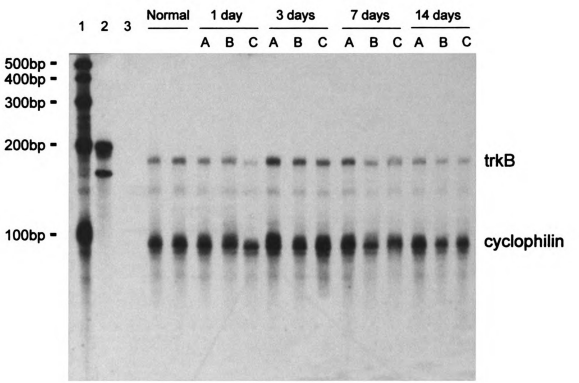

Figure 4-2. The mRNA for full-length trkb was detected using RNase protection assay. Total RNA from rat retina was extracted and hybridized with biotin labeled probes for full-length trkB and cyclophilin (used as internal control). Samples were digested by RNase A1/T and separated in polyacrylamide urea gel. Electrophoresis was followed by electroblotting of RNA to nylon membranes. RNA was visualized by chemiluminesence. The protected fragment of trkB and cyclophilin are 185bp and 103bp, respectively. Lane 1: size marker; lane 2: full length probe, without digestion; lane 3: full length probe, no sample, digestion control; A: BDNF application only; B: optic nerve crush only; C: optic nerve crush and BDNF application.

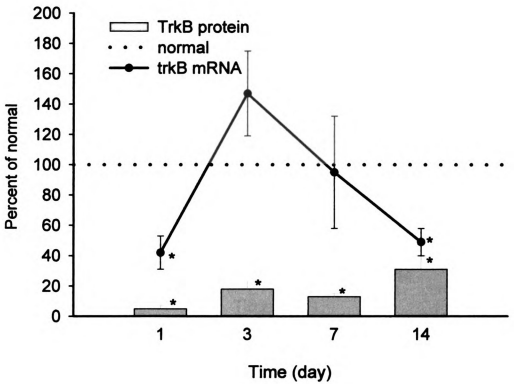

Figure 4-3. Changes of full-length TrkB protein and mRNA following BDNF application in normal rat retina. Relative protein levels were determined by Western blot and relative mRNA levels were determined by RNase protection assay. * $p<0.05$, compare to normal (100%). One sample t-test with Bonferroni's adjustment. Error bars are ± SD.

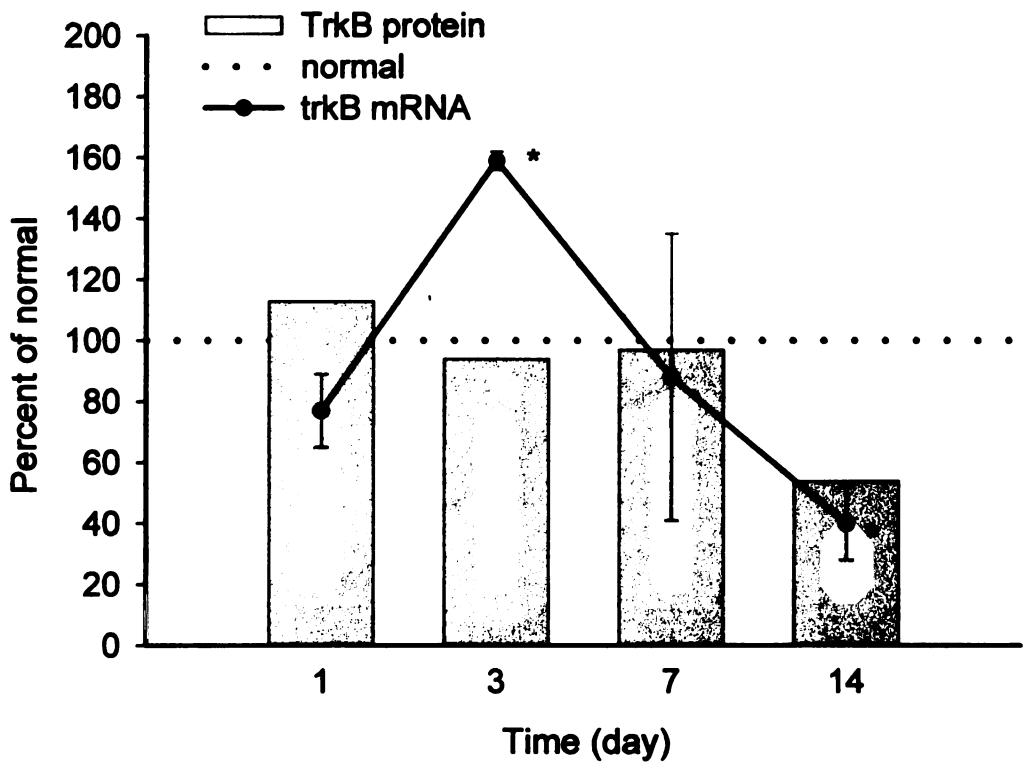

Figure 4-4. Changes of full-length TrkB protein and mRNA following opitc nerve crush in adult rats. Relative protein levels were determined by Western blot and relative mRNA levels were determined by RNase protection assay. * $p<0.05$ for trkB mRNA, compare to normal (100%). One sample t-test with Bonferroni's adjustment. Error bars are ± SD.

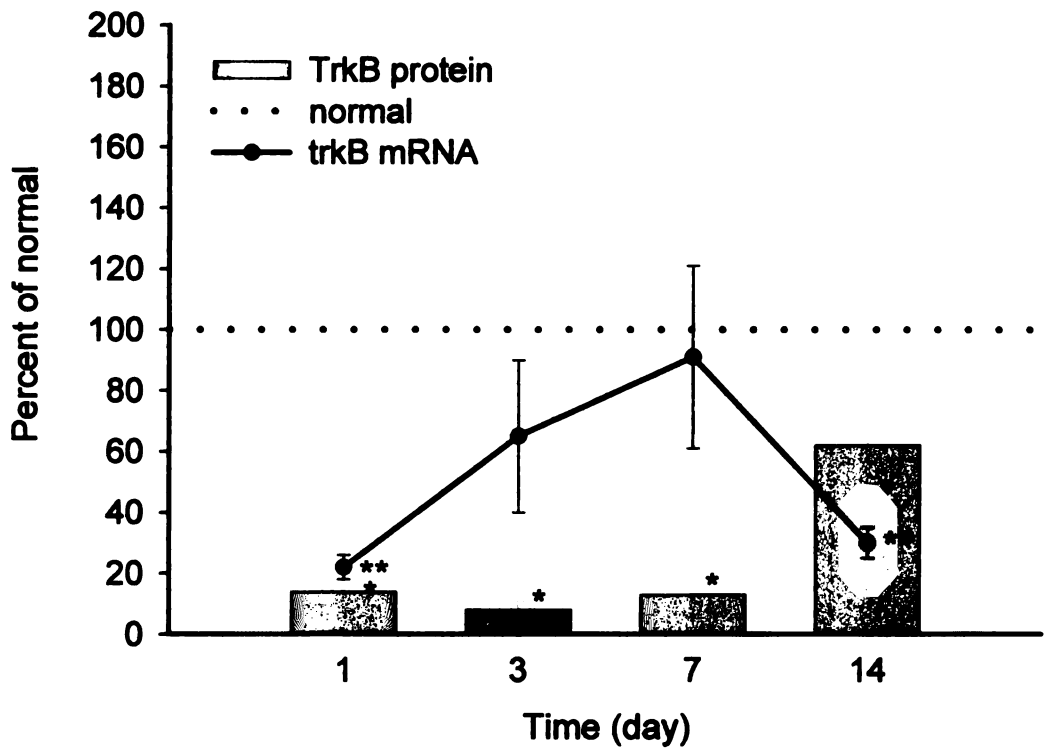

Figure 4-5. Changes of full-length TrkB protein and mRNA following BDNF application in adult rats received optic nerve crush. Relative protein levels were determined by Western blot and relative mRNA levels were determined by RNase protection assay. * $p<0.05$ for trkB mRNA, compare to normal (100%). ** $p<0.05$ for TrkB protein, compare to normal (100%). One sample t-test with Bonferroni's adjustment. Error bars are ± SD.

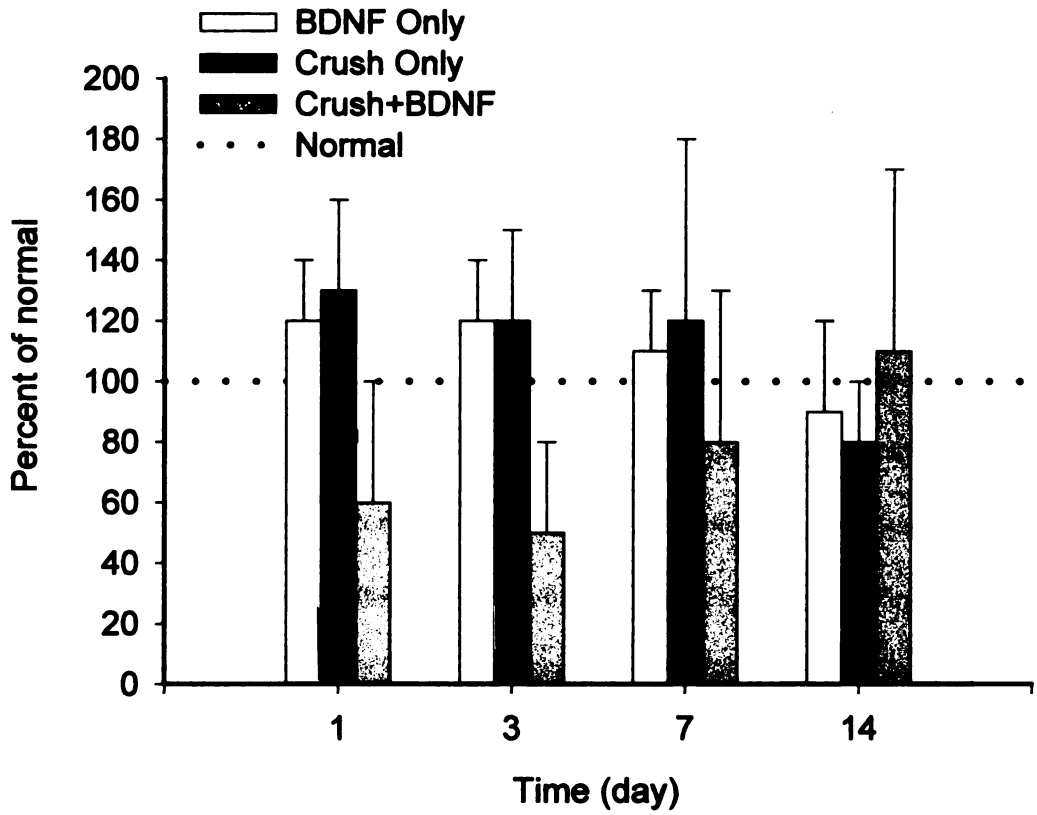

Figure 4-6. Changes of truncated TrkB protein in the rat retina was not statistically insignificant following optic nerve damage and/or BDNF application, as determine by Western blot analysis. Error bars are ± SD.

Epilogue

Glaucoma is a disease of the visual system that is estimated to affect approximately 65 million people worldwide. Most cases of glaucoma are asymptomatic; pressure within the eye rises slowly and the disease progresses undetected until visual impairment is noticed. Studies indicate that pressure-induced damage to the optic nerve and retrograde degeneration of ganglion cells in the retina itself are common occurrences of glaucomatous neuropathy. While increasing public awareness and improving early detection techniques is important, better treatment strategies also are needed.

At present, the primary treatment for glaucoma remains reduction of intraocular pressure (IOP). While this approach has been shown to slow progression of the disease, it rarely is beneficial in preventing further neuronal degeneration. Is there a better treatment strategy? One possibility is to combine a reduction in IOP with the application of a neuroprotectant to the glaucomatous eye. Previous studies have shown that a particular class of proteins called neurotrophins can trigger intracellular signaling pathways that promote neuronal survival following injury. Of the different types of neurotrophins, brain derived neurotrophic factor (BDNF) has been shown to be a strong neuroprotectant in the rat eye, and our initial studies confirm that conclusion. However, differences in the general anatomy and physiology of the rat eye and retina suggest that one must use caution when attempting to relate the results of rat studies to primates. Thus, as an intermediate step to testing the neuroprotective effects of BDNF in primates, we conducted a series of experiments where we investigated the effectiveness of BDNF to preserve retinal ganglion cells in cats following optic nerve injury. The cat eye is similar to the primate eye in size and volume, their retinae contain similar types of ganglion

cells, and the central organization of the pathway also is similar. Although a much larger amount of BDNF was needed for the cat versus the rat, these studies indicated that BDNF is an effective neuroprotectant in primate-sized eyes. Interestingly, we found that increasing the level of applied BDNF did not result in a concomitant increase in ganglion cell survival, but in fact resulted in a lower level of neuroprotection. This led us to ask what might be limiting the effectiveness of BDNF. Since high levels of BDNF resulted in a strong inflammatory response, and previous work has shown that inflammatory cells such as macrophages and leukocytes are capable of producing nitric oxide and various cytokines, we asked whether blocking the inflammatory response might be more beneficial to ganglion cell survival. Combining the BDNF-treatment with daily injections of the nitric oxide blocker L-NAME was effective in blocking the inflammatory response, but did not result in any greater level of neuronal survival. Thus, it doesn't appear that elevated levels of nitric oxide, which can be neurotoxic, underlie the limited neuroprotective effects of BDNF in the retina. The possible negative role of cytokines remains to be tested.

A second possible limiting factor we considered was whether the optic nerve injury had an effect on the non-neuronal support cells of the retina. Müller cells are the primary glial cells of the retina. Their cell bodies are located in the middle of the retina and the endfeet of their bipolar processes form the inner and outer limiting membranes of the retina. Müller cells play an important role in maintaining retinal homeostasis by controlling ion and gluatamate levels in the retina. Previous studies have indicated that ganglion cells can be damaged by gluatamate-induced excitotoxicity. Glial fibrillary acidic protein (GFAP) was used as an indicator of Müller cell activity in response to the

nerve injury, and glutamine synthetase (GS) was used as a marker to determine whether there was a loss of Müller cells in the injured retina. The data indicated that optic nerve crush did not result in a loss of Müller cells, and that they were highly reactive in response to the injury. In addition, the GS work indicated the ability of these cells to rapidly shift their levels of this protein from the cell body to the inner retina near the ganglion cells following optic nerve injury. Presumably, this shift occurs as a protective response to the increase in glutamate released by dying ganglion cells. While BDNF had no effect on GS or GFAP levels, it would be of interest to know whether greater ganglion cell survival might be achieved by combining application of BDNF with the overexpression of GS by Müller cells.

TrkB is the high affinity receptor used by BDNF to initiate intracellular signaling pathways, including those involved in neuronal survival. In vitro and in vivo studies have indicated, however, that BDNF also results in a down regulation of the TrkB receptor protein. As a third possible explanation for the reduced level of ganglion cell survival with increased levels of BDNF, we asked whether BDNF might limit its own effectiveness in the retina by decreasing the level of its high affinity receptor. Western blot analyses of retinas from eyes that received only injections of BDNF showed that BDNF does indeed have a dramatic effect on TrkB levels in the retina in vivo. In fact, only 5% of this high affinity receptor is present the day after the injection. This significant reduction most likely explains, at least in part, why in the cat studies we did not achieve greater ganglion cell survival with increased levels of the drug, or why multiple injections yielded no better result than that seen following a single injection.

These data are important for future studies aimed at determining an effective time course of BDNF treatment.

The reduction in TrkB receptor most likely results from the natural sequence of events used by cells to remove receptors following activation. The internalized TrkB receptor is tagged by ubiquitin, which then makes it a target for degradation by proteosomes. How does this degradation process affect the production of new receptors? To answer this question, we used RNase protection assay to examine the effect that BDNF also has on the levels of trkB mRNA in the retina. Depending on when we looked, we found both reductions and elevations in mRNA levels, and these changes were not always well matched with changes in TrkB protein. While significantly more work is needed to determine the relation between BDNF, trkB and TrkB, it also is important to note that the data are based on the analysis of whole retina. Thus, discrepancies between protein and mRNA levels might reflect different expression profiles of the various cells within the retina. Future studies need to aim at isolating specific classes of ganglion and glial cells and examining their responses to BDNF independently.

In summary, the results of these experiments provide encouraging data indicating that BDNF is a potential neuroprotectant for use in glaucoma patients. When combined with control of intraocular pressure, it might preserve a greater level of vision by helping injured neurons recover, rather than proceed to an apoptotic death. The studies also indicate, however, that future work aimed at enhancing the effectiveness of BDNF as a retinal neuroprotectant must consider a combination of approaches, such as combining BDNF with blockers of glutamate excitoxicity, TrkB overexpression, GS overexpression, or neurotrophins that stimulate neurons using different receptor mechanisms.