CNTF and BDNF Have Similar Effects on Retinal Ganglion Cell Survival but Differential Effects on Nitric Oxide Synthase Expression Soon after Optic Nerve Injury

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PURPOSE. To investigate the effect of ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) on retinal ganglion cell (RGC) survival and nitric oxide synthase (NOS) expression in the retina during the early phase of optic nerve (ON) injury, and to examine whether intraperitoneal application of the NOS scavenger nitro-L-arginine (L-NA) could protect the injured RGCs.

METHODS. RGCs were retrogradely labeled with granular blue 3 days before the ON was intraorbitally transected. RGC survival was examined 1 week after ON transection and intraocular injection of CNTF and/or BDNF, or 1 to 2 weeks after daily intraperitoneal injection of the NOS inhibitor L-NA. NOS expression was examined by NADPH-diaphorase histochemistry and neuronal NOS (nNOS) immunohistochemistry, and nNOS-positive cells were identified by various staining approaches.

RESULTS. Both CNTF and BDNF significantly increased RGC survival 1 week after ON injury. In the ganglion cell layer (GCL), CNTF did not increase the number of NADPH-diaphorase positive (+) cells but appeared to reduce the intensity of NADPH-diaphorase staining, whereas BDNF increased the number of NADPH-diaphorase (+) cells and also appeared to enhance the intensity of NADPH-diaphorase staining. In the GCL, amacrine cells but not RGCs were nNOS (+). Some macrophages were also nNOS (+). In contrast, no amacrine cells were nNOS (+) in the inner nuclear layer. Daily intraperitoneal injection of L-NA at appropriate concentrations promoted RGC survival for 1 or 2 weeks after ON injury.

CONCLUSIONS. Both CNTF and BDNF protected RGCs after ON injury. CNTF and BDNF acted differently on NOS expression in the GCL. Intraperitoneal injections of L-NA at appropriate dosages enhance RGC survival. (Invest Ophthalmol Vis Sci. 2005; 46:1497–1503) DOI:10.1167/iovs.04-0664

Blindness is often a result of traumatic injury to the optic nerve (ON) and pathologic conditions such as glaucoma, diabetes, Leber's congenital ON atrophy, retinitis pigmentosa, and ischemic optic injury.1–7 ON injury often results in rapid retinal ganglion cell (RGC) death via apoptosis. When the optic axons were injured close to the eye, RGCs survived for the first 3 days in hamsters or the first 4 to 5 days in rats, and then died abruptly in large numbers, reducing the RGC population to <10% of the original population on day 14.8,9 Ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) have been widely shown to protect RGCs after injury.10–13 We recently showed that CNTF promotes RGC survival and axonal regeneration via JAK/STAT3, PI3K/akt, and MAPK/ERK pathways.14,15

Nitric oxide synthase (NOS) has been found in microglia, amacrine cells, and Müller cells but not in RGCs.16–18 NOS plays a critical role in axotomy-induced RGC death.16,17 Although BDNF is one of the most effective neurotrophic factors in promoting RGC viability, it has also been found to upregulate NOS by increasing the activity of NADPH-diaphorase, a reliable marker for NOS.18,19 Thus, when an intravitreal injection of BDNF was combined with the systemic administration of an NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME), a synergistic effect on RGC protection was observed.20 However, it is currently unknown what effect CNTF has on NOS activity in the retina and whether it has a protective effect on RGC viability soon after ON injury. In this study, the immediate protective effect of CNTF and/or BDNF was examined 1 week after ON axotomy. Then, the NADPH-diaphorase activity in the retina after CNTF and/or BDNF treatment was investigated at this time point. Using double-immunohistochemistry and double-labeling approaches, we also identified what types of cells in the retina expressed NOS. Finally, we investigated whether intraperitoneal application of the NOS scavenger nitro-L-arginine also protects RGCs after ON injury and whether it provides the protection in a dose-dependent manner.

METHODS

Six to 8-week-old young adult hamsters were used in the study. This study was performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The surgical procedure was also approved by the Animal Ethics Committees of Shantou University Medical College and The University of Hong Kong. Animals were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (Nembutal, 60 mg/kg body weight; Rhone Merieux, Pikenba, NSW, Australia) for all surgical procedures.
Prelabeling of RGCs and ON Transection

RGCs were retrogradely labeled by applying the fluorescent dye, granular blue (GB; Sigma-Aldrich, St. Louis, MO) to the superior colliculi (SC) as previously described in hamsters. In brief, labeling was achieved by applying small pieces of Gelfoam (Pharmacia & Upjohn, Uppsala, Sweden) soaked in 3% GB to the surfaces of both SCs, after cortical tissues and the pia overlaying the SC had been carefully removed. Care was taken not to damage the SCs. After an interval period of 3 days, the left intraorbital ON was transected under an operating microscope in all animals, as previously described. The left ON was exposed through a superior temporal intraorbital approach, and transected 1 mm from the optic disc using a pair of small iris scissors, taking care to avoid any damage to the ophthalmic artery underneath.

Experimental Groups

Animals were divided into the following experimental paradigms:

1. RGC prelabeling → ON transection → effect of CNTF and/or BDNF on RGC survival 1 week after ON injury
2. ON transection → effect of CNTF and/or BDNF on NOS expression 1 week after ON injury
3. RGC prelabeling → ON transection → effect of the NOS inhibitor nitro-arginine (L-NA) on RGC survival 1 or 2 weeks after ON injury

Immediately after the ON was transected, animals in each paradigm were divided into various experimental groups:

**Experiment A.** The effect of CNTF and/or BDNF on RGC survival 1 week after ON injury was studied in this part. Experimental groups were: (1) vehicle (saline) control (n = 6); (2) low-dosage CNTF treatment (0.5 μg/2 μL, n = 6; Peprotech, Rehovot, Israel); (3) high-dosage CNTF treatment (2 μg/2 μL; n = 6); (4) low-dosage BDNF treatment (1.25 μg/2 μL, n = 4; Peprotech); (5) high-dosage BDNF treatment (5 μg/2 μL; n = 4); (6) a combination of low-dosage CNTF and BDNF treatments (0.5 μg/2 μL CNTF and 1.25 μg/2 μL BDNF; n = 6), and (7) a combination of high-dosage CNTF and BDNF treatments (2 μg/2 μL CNTF and 5 μg/2 μL BDNF; n = 5). The low dosages of CNTF and BDNF, which had been shown to give a half-maximum effect, coupled with the high-dosage approaches, were used to examine whether there was a dose-dependent or additive effect of these two neurotrophic factors. Normal animals in which animals did not receive any surgery (n = 8) served as control subjects.

**Experiment B.** The effect of CNTF and/or BDNF on NOS activity 1 week after ON injury was studied in this part. Experimental groups were the same as for Experiment A, except for the number of animals involved: (1) vehicle (saline) control (n = 8); (2) low-dosage CNTF treatment (n = 5); (3) high-dosage CNTF treatment (n = 6); (4) low dosage BDNF treatment (n = 5); (5) high dosage BDNF treatment (n = 6); (6) a combination of low-dosage CNTF- and BDNF treatments (n = 5); and (7) a combination of high-dosage CNTF- and BDNF treatments (n = 6). Normal animals in which animals did not receive any surgery (n = 6) served as control subjects.

In both paradigms A and B, the chemicals were injected into the vitreous humor of the eye. Second injections were administered on day 5 after ON transection.

**Experiment C.** The effect of intraperitoneal injections of the NOS scavenger t-NA (cat. no. N10403; Aldrich Chemical Co., Milwaukee, WI) on RGC viability was studied at both 1 and 2 weeks after ON injury. To investigate whether t-NA protects RGCs in a dose-dependent manner, we used various concentrations (1.5, 3, 4.5, and 6 mg/kg per day) of t-NA. Four animals were used in each treatment group. The number of RGCs in normal intact animals (n = 8) served as the control.

Animals were allowed to survive in a room with a 12-hours dark-light cycle. They were eventually killed by an overdose of intraperitoneally injected pentobarbital sodium and were perfused transcardially with cold saline for 5 minutes followed by 4% paraformaldehyde for 10 minutes. The left eyeballs were removed and the retinas were dissected. The pigment layer of each retina was removed gently with a brush.

**Counting of GB-Labeled RGCs**

For RGC survival experiments, retinas were postfixed in 4% paraformaldehyde for 1 hour, flattened on gelatin-coated slides, and coverslipped with 30% glycerol (Merck, Darmstadt, Germany). The retinas were examined under a fluorescence microscope (model E1000; Nikon, Tokyo, Japan) with a blue-violet excitation filter. Labeled RGCs were counted in 30 to 40 sample fields (each occupying 200 μm²). A grid was placed on each retinal image on the monitor, and fields at each grid intersection point were chosen for RGC counting. The mean density of labeled RGCs in each retina was then multiplied by the area of the retina to obtain the estimated number of labeled RGCs in the whole retina.

**NADPH-Diaphorase Histochemistry**

For NADPH study, the retinas were immersion fixed in 4% paraformaldehyde and 0.2% picric acid (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4) for 30 minutes and washed with 0.1 M Tris buffer (pH 8.0) four times for 15 minutes each time. The expression of NOS was first detected by NADPH-diaphorase histochemistry. Retinas were incubated at 37°C for 30 minutes in 10 ml 0.1 M Tris-HCl (pH 8.0) containing 10 mg NADPH (Sigma-Aldrich), 2.5 mg nitroblue tetrazolium (Sigma-Aldrich; dissolved in 50 μl dimethylsulfoxide [DMSO]), and 20 μl Triton X-100. After a rinse in Tris buffer, retinas were flattened, air dried, dehydrated, and coverslipped. Then, the number of NADPH-diaphorase positive (') cells in the ganglion cell layer (GCL) was counted and the density obtained.

Two retinas from each group were removed after perfusion with saline. The eye cups without cornea and lens were immersion fixed in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) for 30 minutes, followed by immersion in 30% sucrose in 0.1 M phosphate buffer overnight at 4°C. Ten-micrometer cryostat sections were cut and collected on gelatin-coated slides and air dried. NADPH-diaphorase histochemistry was performed on these sections, as described earlier.

**NOS Immunohistochemistry**

Sections were incubated in primary antibody raised from rabbit against neuronal NOS (nNOS; BD Biosciences, San Diego, CA) and inducible NOS (iNOS; BD Biosciences) at 1:200 in 0.1 M Tris buffer overnight at 4°C. After three washes in Tris buffer, the sections were incubated in goat anti-rabbit secondary antibody overnight at 4°C, followed by the avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) reaction. The specificity of the nNOS antibody was tested with the pituitary gland used as the positive control and the omission of primary antibody used as the negative control. Specific labeling was visualized with diaminobenzidine (DAB) staining, and the number of positive neurons in the GCL was counted under a microscope.

**Double Staining to Identify NADPH-Diaphorase and nNOS-Positive Cells in the Retina**

To identify which cells expressed NADPH-diaphorase or NOS, double immunohistochemical staining using rabbit anti-nNOS antibody (BD Biosciences) paired with mouse anti-BII tubulin (TUJ1 antibody for RGCs; Babco, Evanston, IL) anti-CD68 (ED1 antibody for macrophages; Serotec, Oxford, UK), or anti-parvalbumin antibodies (antibody for amacrine cells; Swant, Bellinzona, Switzerland) was performed in retinal sections and wholemounts after saline, CNTF, and BDNF treatments (n = 3 each group). For each retina in this part of the study, half the retina was cryosectioned and the other half wholemounted. Briefly, retinal sections or wholemounts were washed three times in PBS and...
blocked in 10% normal goat serum (NGS) and 0.2% Triton for 1 hour. Primary rabbit antibody against nNOS (1:500) together with mouse TUJ1 (1:400), ED1 (1:500), or anti-parvalbumin (1:400) antibodies were added into the blocking solution and incubated overnight at 4°C. After three 10-minute washes in PBS, retinal sections were incubated with fluorescein-5-isothiocyanate (FITC)–conjugated anti-mouse IgG (1:400; ICN, Aurora, OH) and Cy3-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies at room temperature for 1 hour. Specificity of immunostaining was assessed by comparing with the negative (no primary antibody) and positive (rat pituitary gland) controls as instructed in the antibody information sheet.

To further confirm whether RGCs were NADPH-diaphorase, double-labeling approaches using fluorescent gold (FG; Fluorogold; Fluorochrome, Englewood, CO) for retrograde labeling of RGCs and NADPH-diaphorase staining RGCs were also performed. Both FG and GB were initially used, but FG labeling was retained well after NADPH-diaphorase histochemistry procedure, thus FG was chosen for this part of the study. RGCs were retrogradely prelabeled by applying 4% FG in both superior colliculi of two animals, and the retinal wholemounts were later stained for NADPH diaphorase. Examination of the staining was performed under normal light (for NADPH) and fluorescent light (for FG).

Statistical Analysis
Data were analyzed with the Dunnett or Bonferroni tests after one-way analysis of variance (ANOVA). The Dunnett test was used to compare mean data of the experimental groups against the control group (saline-treatment group), and the Bonferroni test was used to compare mean intergroup data (e.g., comparisons between low- and high-dosage groups with CNTF, BDNF, and CNTF/BDNF treatments).

RESULTS
Protection of RGCs by CNTF and/or BDNF
Typical appearance of GB-labeled RGCs is shown in the fluorescence photomicrographs in Figure 1. RGCs contained relatively uniform GB labeling with oval or round somata. ON axotomy at 1 mm behind the eyeball resulted in a dramatic loss of RGCs 1 week after injury. The average number of GB-labeled viable RGCs in the saline-treated group was 8880 ± 3949 cells/retina (mean ± SD; Fig. 2A). Note that there was an average of 69,166 RGCs/retina in normal intact hamsters (Fig. 2A); thus, a large majority of RGCs died after the ON injury. At this time point, RGC protection by CNTF and BDNF was clear. The average number of GB-labeled viable RGCs after CNTF treatments at low and high dosages was 19,776 ± 3,067 and 27,957 ± 4,076 cells/retina, respectively, and after BDNF treatment at low and high dosages was 18,809 ± 2,819 and 25,773 ± 4,969 cells/retina, respectively (Fig. 2A). Although there was a trend that treatment with the higher dosage of neurotrophic factors yielded a higher number of surviving RGCs, all neurotrophic factor treatments significantly increased the number of surviving RGCs (P < 0.001–0.05, Dunnett test; Fig. 2A). A significantly higher number of surviving RGCs was seen in the high-dosage CNTF treatment group compared with the low-dosage CNTF group (P < 0.05, Bonferroni test; Fig. 2A). There was no obvious synergistic effect when CNTF and BDNF were applied together at either low or high dosage. The average number of GB-labeled RGCs was only slightly higher after combined CNTF and BDNF treatments at low (27,994 ± 3,445 cells/retina) and high (31,386 ± 6,436 cells/retina) dosages (Fig. 2A).

Figure 1. Fluorescence photomicrographs showing GB-labeled viable RGCs in normal (A), ON transected (B), and CNTF/BDNF-treated (C) retinas. Scale bar, 50 μm.

Figure 2. Average number of GB-labeled viable RGCs (A) and mean density of NADPH-diaphorase+ cells in the GCL (B) under various conditions. Error bars, SD. A comparative analysis was made against the saline group with the Dunnett test, except as specified in the text for intergroup comparison with Bonferroni test. Significance level, *P < 0.05, **P < 0.01; ***P < 0.001.
Effect of CNTF and/or BDNF on NADPH-Diaphorase Activity

Positive staining of NADPH-diaphorase appeared in blue in all retinas. There were several types of cells that were NADPH-diaphorase+ in the retinas, mainly in the GCL (Figs. 3A, 3D, 3G, 3J, 3M; green arrows in 3B, 3E, 3H, 3K, 3N), the inner nuclear layer (INL; yellow arrows in Figs. 3B, 3E, 3H, 3K, 3N), and pigment epithelial cell layer (ECL; Fig. 3B). Cells in these layers were also nNOS+ (Figs. 3C, 3F, 3I, 3L, 3O). These cells in the INL usually gave rise to a single process that descended vertically or obliquely toward the inner plexiform layer (IPL) and ramified. NADPH-diaphorase+ (Figs. 3B, 3E, 3H, 3K, 3N) and nNOS+ (Fig. 3C, 3F, 3I, 3L, 3O) fibers were densely distributed in the IPL laminae. The iNOS immunostaining was not satisfactory, owing to the high background (data thus not shown). The number of NOS-expressing cells labeled by nNOS immunohistochemistry appeared to be less than that in NADPH-diaphorase immunohistochemistry. In the outer plexiform layer (OPL), there were some weakly NADPH-diaphorase+ cells that had very small cell bodies (Figs. 3B, 3E, 3H, and 3K, red arrowheads), and no such cells were detectable by nNOS immunohistochemical staining (Fig. 3C, 3F, 3I, 3L, 3O).

The density of NADPH-diaphorase+ neurons in the GCL was 301 ± 32 neurons/mm² (n = 6) in the normal hamsters. This number decreased significantly to 85.6 ± 10.9 neurons/mm² in the saline control group after ON injury. Thus, ON axotomy appears to affect the number of NADPH-diaphorase+ neurons in the GCL. After intraocular CNTF treatment at low dosage, this density was increased to 123 ± 6.1 neurons/mm² (n = 5), which further increased to 312 ± 49 neurons/mm² after high-dosage treatment (n = 6). Whereas these amounts after CNTF treatments were not significantly different from the number in the saline control, the number in the high-dosage group was significantly higher (P < 0.001) than that in the low-dosage group (Fig. 2B).

The average density of NADPH-diaphorase+ cells in BDNF-treated retinas was 159 ± 26 cells/mm² (n = 5) at low-dosage and 395 ± 42 cells/mm² (n = 6) at high-dosage treatment. Whereas the number in the low-dosage group was not significantly different from that in the saline control, the number in the high-dosage group was significantly higher than in both the saline control (P < 0.001; Dunnett test) and the low-dosage group (P < 0.001; Bonferroni test; Fig. 2B), indicating that BDNF at appropriate concentrations increases NADPH activity in the GCL.

Combined treatment of BDNF and CNTF yielded an average number of NADPH-diaphorase+ cells of 256 ± 14 cells/mm² (n = 5) at low dosages and 400 ± 24 cells/mm² (n = 6) at high dosages (Fig. 2B). No synergistic effect on the density of NADPH-diaphorase+ cells was seen after either low- or high-dosage combination treatment because these treatments did not result in a significant increase in the density of NADPH-diaphorase+ cells when compared with individual neurotrophic factor treatment groups with the same dosage (Fig. 2B). However, when the low- and high-dosage treatments of the same neurotrophic factor were compared against each other, a significant increase in the density of NADPH-diaphorase+ cells was seen in all treatment groups (CNTF, P < 0.001; BDNF, P <
A clear increase in the intensity of NADPH-diaphorase staining was also seen when BDNF was used, irrespective of CNTF application (Figs. 3J, 3M, 3K, 3N).

Identification of NADPH-Diaphorase Positive Cells in the Retina

NADPH-diaphorase+ cells were neuronal in appearance (round or oval cell body with neurites) in the GCL (Fig. 4A). The specificity of the nNOS antibody was confirmed by both positive (Fig. 4B; pituitary gland) and negative (omission of primary antibody; data not shown) immunostaining. Double immunohistochemical staining with the nNOS antibody and TUJ1 (for RGCs) showed that RGCs were not TUJ1+ but were nNOS+ in the GCL (E, F). Some ED1+ macrophages in the GCL (G) were nNOS+ (H). Scale bar, 50 μm.

Effect of the NOS Scavenger L-NA on RGC Survival

NOS inhibitor L-NA was used to examine whether intraperitoneal application of this NOS inhibitor was able to prevent RGC death soon after ON injury. We found that 1 week after ON axotomy, approximately 75% of RGCs were lost; the number of viable GB-labeled RGCs was 15,980 ± 3,093 cells/retina (Fig. 5A). Daily intraperitoneal injections of L-NA at 1.5, 3, 4.5, and 6 mg/kg all significantly enhanced RGC viability (24,292 ± 3,247, 29,484 ± 4,838, 35,728 ± 5,006, and 31,723 ± 4,841 cells/retina, respectively) 1 week after ON injury, with the concentration of 4.5 mg/kg being the most effective (Fig. 5A).

Similar observations were also obtained 2 weeks after ON axotomy. In ON lesion-only animals, only 1370 ± 900 GB-labeled RGCs/retina survived 2 weeks after ON axotomy (Fig. 5B). The number increased to 2696 ± 823 cells/retina after daily L-NA treatment at a 1.5-mg/kg concentration, 4234 ± 483 cells/retina at 3 mg/kg, 5250 ± 545 cells/retina at 4.5 mg/kg, and 3428 ± 339 cells/retina at 6 mg/kg (Fig. 5B). Compared
with the lesion-only group, L-NA at 3, 4.5, and 6 mg/kg, but not at 1.5 mg/kg, significantly enhanced RGC viability 2 weeks after ON axotomy (Fig. 5B). Again, L-NA at 4.5 mg/kg was the most effective dosage in protecting RGCs (Fig. 5B). These results demonstrate that, apart from local application of the NOS scavenger, a systematic approach of the NOS blockade can also protect neuron viability soon after injury.

**DISCUSSION**

In this study we found that both CNTF and BDNF protected RGCs soon after ON injury. Although BDNF had been widely shown to protect RGCs under various conditions, the immediate effect of CNTF on RGC viability was unknown. In the present study, CNTF also significantly promoted RGC survival soon after ON injury. The extent of CNTF protection on RGCs was similar to that rendered by BDNF 1 week after ON injury. However, whereas both CNTF and BDNF protected RGCs, they had different effects on NOS activity in the retina. CNTF did not affect the number of NADPH-diaphorase− cells and appeared to reduce the intensity of NADPH-diaphorase staining whereas BDNF upregulated NOS activity in both the number and intensity of NADPH-diaphorase− cells. Although NADPH-diaphorase histochemistry and immunohistochemistry are not a reliable means of determining the changes in intensity of NADPH-diaphorase− cells, the differential effects of CNTF and BDNF on the proportion of NADPH-diaphorase− cells suggest that different mechanisms of CNTF and BDNF were involved in protecting RGCs.

The activity of NOS was detected with NADPH-diaphorase histochemistry, which is a reliable marker for NOS activity. In retinas, there are three types of NOS that can be detected in different populations of cells: (1) nNOS, which releases a relatively low level of NO for short periods and is commonly localized in neurons in brain; (2) iNOS, which synthesizes a high level of NO for extended periods and is mainly found in macrophages, activated microglia cells, astrocytes, and Müller cells in the retina; and (3) eNOS, which synthesizes endothelial NO in the endothelial cells. Both nNOS and iNOS have been reported to be involved in the death of axotomized RGCs. In the present study, the survival of axotomized RGCs was associated with both the inhibition of NOS activity and the supply of neurotrophic factors.

The number of NADPH-diaphorase− cells in the retinal wholemounts did not change significantly after intraorbital ON transection. A similar observation was reported previously. We noted that NOS activity is present only in displaced amacrine cells but not in RGCs in the GCL. Using various staining approaches, we confirmed these observations in this study. Thus, the increased number of NADPH-diaphorase− cells in the GCL after BDNF or BDNF/CNTF treatment indicates an upregulation of NADPH-diaphorase expression in amacrine cells. This means that after BDNF treatment, there are more amacrine cells expressing NADPH that normally do not express it, which is consistent with previous reports that BDNF increases the number of NADPH-diaphorase− cells in the adult rat retina. However, localization of NOS activity in the retina has not been reported consistently in the literature. Although we did not find NOS activity in RGCs in hamsters and similar findings were also reported in rats, other studies have shown NOS activity in rat RGCs, especially after ON injury. The species difference may underlie the different observations in NOS localization and activity between hamsters in our study and rats in others’ studies.

The NADPH-diaphorase/NOS activity is highly related to the death of RGCs. This was demonstrated by NOS inhibition studies in which intravitreal application of NOS inhibitors enhanced RGC survival after ON injury. An increase of NO in amacrine cells in BDNF-treated retinas may lead to the death of some axotomized RGCs, because application of NOS inhibitors suppressed this adverse effect of BDNF treatment. These results suggest that intravitreal application of certain drugs can affect other types of cells besides RGCs in the retina, which may in turn exert an adverse effect on the RGCs. The synergistic effect of BDNF and NOS inhibitors clearly indicates that NOS inhibition may block the detrimental effect of BDNF on RGCs by blocking the BDNF-induced increase in NOS activity in amacrine cells. This finding is supported by a study that showed that the death of neighboring cells was caused by the generation of NO in the glutamate receptor overstimulated neurons, whereas the NOS-containing cells themselves were less sensitive to NO toxicity. In the present study, we show that intraperitoneal application of the NOS scavenger L-NA at appropriate dosages can also protect RGCs 1 to 2 weeks after ON injury.

TrkB, the high-affinity BDNF receptor, and NADPH-diaphorase have been found to coexpress in amacrine cells. The colocalization of NADPH-diaphorase staining with TrkB indicates that BDNF may exert an effect on NOS activity via this high-affinity receptor. In contrast, CNTF receptor-α (CNTFRα), one component of the CNTF receptor complex (CNTFRα, LIFβ, and GP130), is expressed in a subpopulation of amacrine cells in the retina. Although CNTF and BDNF act differently on NOS activity in the retina, the combined application of BDNF and CNTF did not achieve a synergistic effect on RGC survival or a diminishing effect on NADPH-diaphorase intensity. These observations suggest that BDNF plays a dominant role over CNTF in RGC protection and NADPH-diaphorase activity. Another mechanism may also exist to explain why the combination of BDNF and CNTF failed to achieve a synergistic effect in promoting RGC survival. For example, it has been shown that both BDNF and CNTF can activate microglial cells. Stimulated microglial cells can release cytotoxic molecules, such as iNOS, which are detrimental to RGCs. The results of this study demonstrate that CNTF and BDNF play different roles in the retina. Although both CNTF and BDNF protect RGCs, CNTF reduces NOS activity whereas BDNF increases NOS activity in the neighboring cells to harm RGCs. The reduction of NOS activity by CNTF may indicate a better potential for RGC protection under certain conditions.

**References**


