Neurotrophin-4/5 (NT-4/5) Increases Adult Rat Retinal Ganglion Cell Survival and Neurite Outgrowth *in vitro*

A. Cohen, G. M. Bray, and A. J. Aguayo*

McGill University Centre for Research in Neuroscience, The Montreal General Hospital Research Institute, Canada, Montréal, Québec, H3G 1A4

SUMMARY

Retinal ganglion cell (RGC) survival and neurite outgrowth were investigated in retinal explants from adult rats. Neurotrophin-4/5 (NT-4/5) caused dose-dependent increases in neurite outgrowth with one-half maximal effects at approximately 0.5 ng/ml and maximal effects at 5 ng/ml. In explants treated for 7 days, the actions of NT-4/5 were similar to those of brain-derived neurotrophic factor (BDNF); with either neurotrophin, nearly twice as many RGCs survived and there was a two- to threefold increase in the number of neurites formed by RGCs. Combinations of saturating concentrations of NT-4/5 and BDNF did not enhance these *in vitro* effects, implying that both neurotrophins share a common signaling pathway. In contrast, nerve growth factor (NGF), neurotrophin-3 (NT-3), or ciliary neurotrophic factor (CNTF) appeared to exert minimal influences on RGC survival or neurite outgrowth.

Keywords: **retinal ganglion cells, neurotrophin-4/5, brain-derived neurotrophic factor, retinal explants, neuron survival.**

INTRODUCTION

Optic nerve injury in adult rodents causes a severe loss of retinal ganglion cells (RGCs) (Grafstein and Ingoglia, 1982; Allcutt et al., 1984; Misantone et al., 1984; Barron et al., 1986; Villegas-Pérez et al., 1993). The death of such injured neurons has been attributed to an interruption of the supply of neurotrophic factors provided by components of their targets and pathways.

Neurotrophin-4/5 (NT-4/5) is a member of the neurotrophin family. Like BDNF, NT-4/5 induces neurite outgrowth from explanted chick dorsal root ganglia but has less effect on nodose ganglia (Hallböök et al., 1991). The two factors are equally effective in evoking rapid tyrosine phosphorylation of TrkB and cell growth in NIH-3T3 or PC12 cells expressing TrkB receptors (Ip et al., 1993), but NT-4/5 provides a greater support than BDNF on the survival of trigeminal neurons (Ibáñez et al., 1993). In addition, transformation assays using NIH-3T3 cells transfected with mutant TrkB receptor indicate that NT-4/5 involves different interactions with TrkB than does BDNF (Ip et al., 1993). While the enhancement of RGC survival by BDNF has been shown *in vitro* (Johnson et al., 1986; Thanos et al., 1989) and *in vivo* (Mansour-Robaey et al., 1994), the effects of NT-4/5 have not been tested on these retinal neurons. In the present study, we have examined the actions of NT-4/5 on adult rat RGC survival and neurite outgrowth *in vitro* and compared its effects to those of BDNF, NT-3, NGF, and CNTF.

Received January 3, 1994; accepted March 8, 1994
Journal of Neurobiology, Vol. 25, No. 8, pp. 953–959 (1994)

CCC 0022-3034/94/080953-07

* To whom correspondence should be addressed.

METHODS

Retinal Explants

At 7 days after crushing one optic nerve intraorbitally to produce a "conditioning" lesion (Ford-Ho-

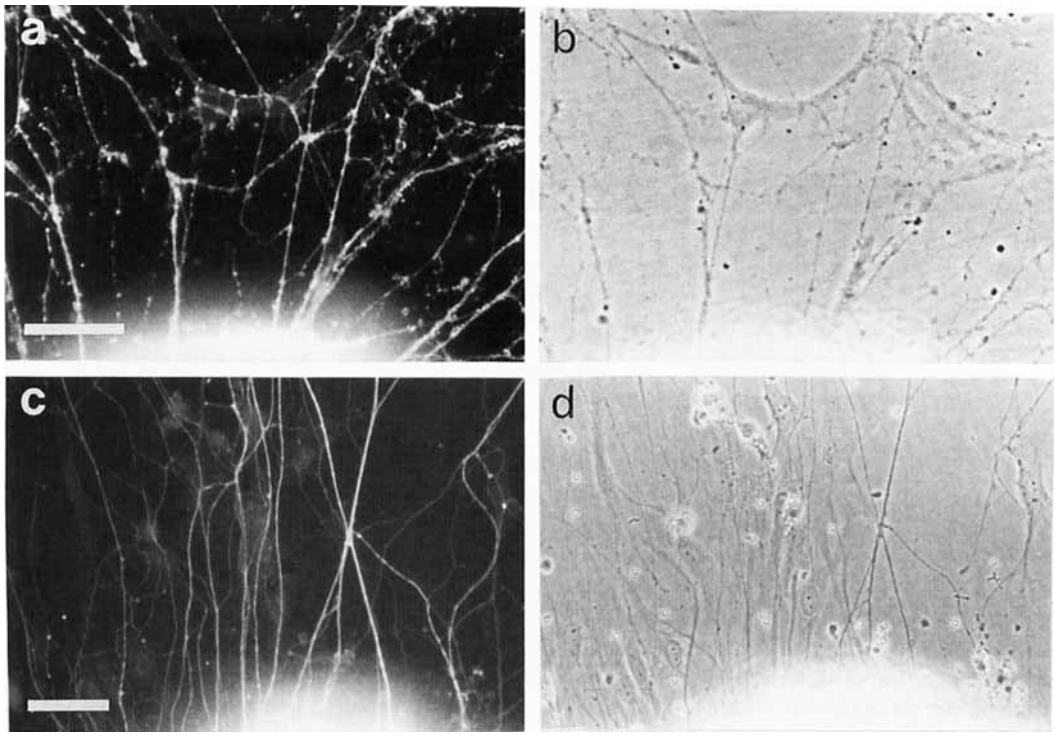

Figure 1 Demonstration of anti-Thy1.1 (a, b) and RT97 (c, d) labelling of neurites that extend from rat retinal explants. Unfixed explants were incubated with Thy1.1 antibodies diluted 1:100, stained with flourescein-conjugated goat anti-mouse antibodies (1:100), and fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer for 20 min at room temperature. For labelling of neurites with RT97, other explants were fixed and permeabilized for 10 min with 100% methanol at −20°C, incubated with RT97 (1:500) for 30 min at 37°C, and immunostained with a fluorescein-conjugated secondary antibody. All neurites that extended from the retinal explants were RT97 as well as Thy1.1 positive, which confirms their RGC origin (Barnstable and Drager, 1984). Panels (a) and (c) are fluorescence photographs and panels (b) and (d) are phase contrast photographs. Scale bar = 50 μm.

levinski et al., 1986; Bähr et al., 1988), retinas from adult female Sprague–Dawley rats were cut into 0.16 mm^2 pieces with a McIlwain tissue chopper. These retinal explants, plated on 35-mm^2 Nunc dishes precoated with poly-L-lysine (Sigma, 200 kDa) and laminin (BRL), were maintained in modified N2 defined medium at 37°C in a 5% CO_2 incubator. Human recombinant NT-4/5 (Regeneron Pharmaceuticals Inc.), rat recombinant BDNF (Regeneron Pharmaceuticals, Inc.), human recombinant NT-3 (Regeneron Pharmaceuticals, Inc.), rat recombinant CNTF (provided by Dr. P. M. Richardson), or mouse NGF (7S NGF, Upstate Biotechnology, Inc.) was added to the culture medium immediately after explant attachment. For counts of RGC survival after explanting, RGCs were retrogradely labelled by applying Gelfoam^R soaked with Fluoro-Gold (FG) solution (2% in 0.9% NaCl containing 10% DMSO) to the surface of both superior colliculi (Vidal-Sanz et al., 1988) 7 days before optic nerve crush and 14 days before explantation.

Examination of Retinal Explants

After incubation for 7 days, the explants were processed for immunocytochemistry with antibodies to rat Thy1.1 (IgG subtype; MRC OX7, Serotec), which recognizes an epitope on the surface of RGCs (Barnstable and Drager, 1984); RT97 (provided by Dr. J. Wood), which recognizes the 200 kDa neurofilament subunit (Anderton et al., 1982); or, TrkB and TrkA (polyclonal antibodies; Santa Cruz Biotechnology Inc.). Fluorescein-conjugated goat anti-mouse antibodies or biotinylated goat anti-rabbit antibodies followed by flourescein-conjugated avidin were used to visualize the primary antibodies. After final washes, all cultures

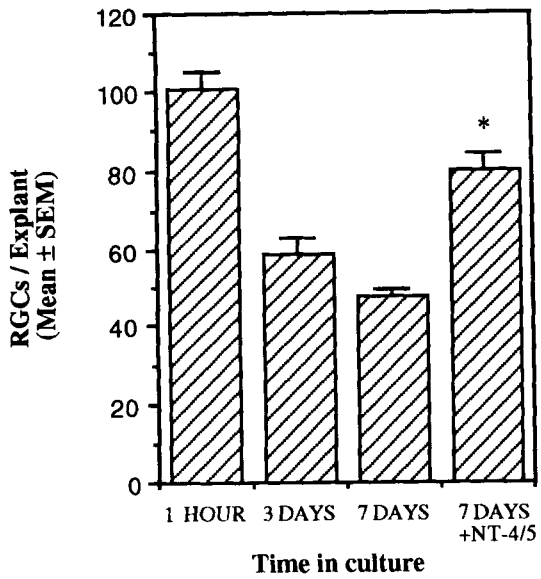

Figure 2 Survival of Fluoro-Gold-labelled retinal ganglion cells (RGCs) *in vitro*. RGCs prelabelled with FG applied to the superior colliculi were counted 1 h, 3 days, and 7 days after explanation. In untreated explants, the number of FG-labelled RGCs decreased with time. The addition of NT-4/5 increased the survival of FG-labelled cells. *Indicates significant difference ($p < 0.01$) from 7 days when analyzed by ANOVA followed by a post-hoc Scheff's test.

were mounted in glycerol/PBS (9:1) containing 22 m*M* of 1,4-diazo-bicyclo-(2,2,2)octane and examined on a Zeiss Axiovert microscope equipped with epifluorescence optics.

Quantitation of RGC Survival and Neurite Outgrowth

Two parameters were measured in number-coded retinal cultures to determine the effect of NT-4/5 on RGCs at 1 week: the number of FG-labelled neurons that persisted in each explant and the number of neurites that extended from each explant. FG-labelled RGCs and RT97-immunostained neurites were counted in well-attached retinal segments with the RGCs facing the substrate, using only retinal explants that measured 0.16–0.26 mm^2 by eye-piece reticule. The RT97 staining, which corresponded to the Thy1.1 immunoreactivity (Fig. 1), was used for counting neurites because its fluorescence was stronger and fewer explants were lost during the staining procedure. Double immunostaining was not possible because both antibodies were mouse IgGs.

Neurites were counted at the edges of the explants where most were unfasciculated. Approximately 2500 explants from eight different experiments were evaluated, and the data were expressed as a percentage of the untreated control group in each experiment. The results were analyzed statistically with a one-way analysis of variance (ANOVA, StatView 512+) followed by a post-hoc Scheff's test.

RESULTS

NT-4/5 Supports RGC Survival and Neurite Outgrowth

RGCs, prelabelled with FG applied to the superior colliculi, were counted to estimate the survival of these neurons *in vitro*. In untreated retinal cultures, there were 101.0 ± 39.3 (Mean ± S.D., $n = 83$) FG-labelled RGCs per explant 1 h after explanting; this number fell to 58.6 ± 26.3 (58%; $n = 40$) by 3 days and to 47.4 ± 16.8 (47%; $n = 54$) by 7 days (Fig. 2). Compared to control explants after 7 days in culture, the addition of NT-4/5 or BDNF increased the survival of FG-labelled RGCs by 1.5- to 2-fold (Figs. 2–4). When the same explants were labelled with RT97, the number of neurites was increased by approximately two- to threefold (Figs. 3, 4).

In a representative group of retinal explants with no added neurotrophin, there were 50.6 ± 24.9 neurites/explant ($n = 38$). In the explants treated with NT-4/5, neurite outgrowth increased by approximately two- to threefold at 7 days. The effect of NT-4/5 was similar to that of BDNF. The average number of neurites formed by explants treated with 50 ng/ml of NT-4/5 was 101.0 ± 30.6 ($n = 31$), while treatments with BDNF produced 117.9 ± 36.3 ($n = 35$) neurites per explant. Treatment with combinations of saturating concentrations (50 ng/ml) of NT-4/5 and BDNF did not further augment the effect of each factor alone (Fig. 4).

The effects of NT-4/5 and BDNF were dose dependent (Fig. 5). The one-half maximal effect of NT-4/5 was observed at a protein concentration of approximately 0.5 ng/ml with the maximal effect at 5 ng/ml, which is similar to values obtained in other systems (Ip et al., 1993; Wong et al., 1993).

Other neurotrophic factors (NT-3, NGF, and CNTF) tested in this *in vitro* system did not have a statistically significant effect on the survival of RGCs or neurite outgrowth from retinal explants (Fig. 4). Nevertheless, CNTF increased RGC survival and NT-3 increased neurite outgrowth by ap-

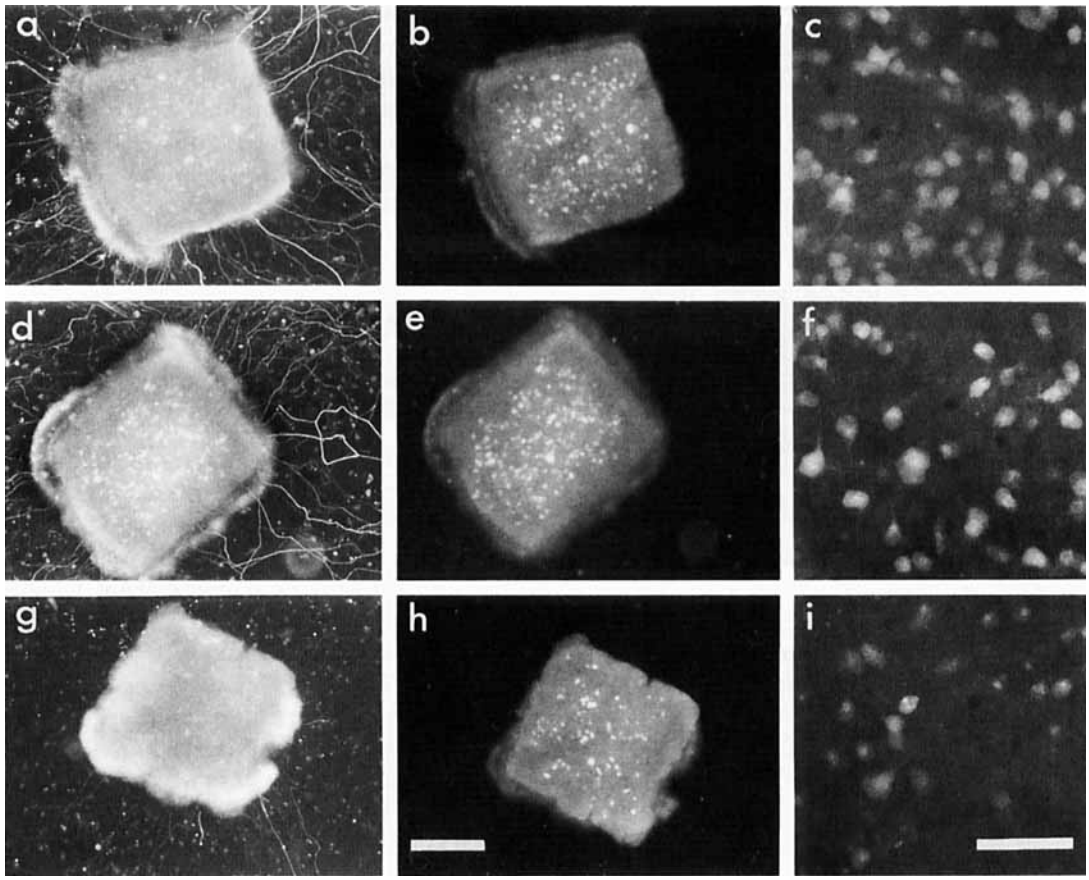

Figure 3 Increased RGC survival and neurite outgrowth in retinal explants treated with NT-4/5 or BDNF. FG prelabelled RGCs, cultured for 7 days in the presence of control medium or medium supplemented with NT-4/5 (50 ng/ml) or BDNF (50 ng/ml), were fixed for 30 min with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 15 min, immunostained with RT97 diluted 1:500, and photographed to visualize RT97 immunolabelling (a, d, g) and FG-labelled RGCs (b, c, e, f, h, i). Representative explants are shown from cultures treated with NT-4/5 (a, b, c), BDNF (d, e, f) or no added trophic factor (g, h, i). The morphology and extent of fasciculation were similar for retinal neurites exposed to NT-4/5 and BDNF. Neurite outgrowth from the retinal explants was observed on the surface of migrating glia (GFAP-positive cells) as well as on laminin. The extent of glial migration from the explants was similar in both control and treated cultures. Scale bar: (a, b, d, e, g, h) 250 μm; (c, f, i) 50 μm.

proximately 50% more than the control cultures. Combinations of saturating concentrations of NT-4/5 or BDNF with the other neurotrophic factors had similar effects to those of NT-4/5 or BDNF alone.

RGC Neurites Express TrkB Receptor

Because the effects of NT-4/5 and BDNF are mediated through the TrkB receptor, we immunostained these cultures with TrkB antibodies. There was strong immunolabelling on neurites that extended from RGCs (Fig. 6) with less intense, punctate labelling on the Müller cells that migrated from the explants. The level of TrkB expression was not affected by incubation with neurotrophin-containing or control medium. When processed with antibodies specific for the TrkA receptors, which binds NGF, no immunolabelling was observed.

DISCUSSION

Neurotrophin-4 (NT-4), which shares 50%–60% amino acid homology with other members of the

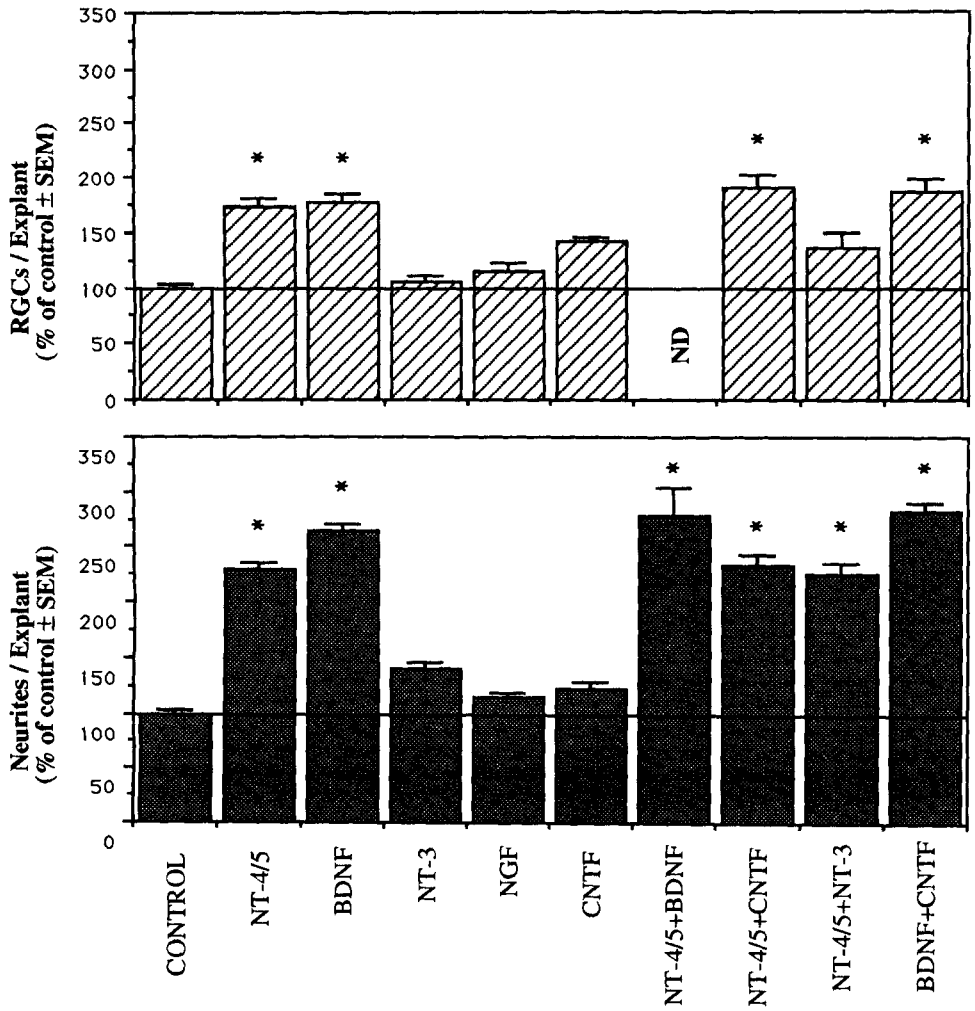

Figure 4 Counts of FG-labelled RGCs (upper panel) and RT97-stained neurites (lower panel) in explants treated with 50 ng/ml of NT-4/5, BDNF, NT-3, NGF, or CNTF. The numbers of RGCs or neurites per explant were counted in three to eight different experiments and normalized to control cultures, which were set at 100%. The hatched bars represent the FG counts and the solid bars represent RT97-stained neurites per explant (n = 40–400). *Indicates significant difference ($p < 0.01$) from control explants when analyzed by ANOVA followed by a post-hoc Scheff's test. *Abbreviation:* ND = not done.

neurotrophin family, was originally isolated from *Xenopus* and viper DNA (Hallböök et al., 1991). The equivalent mammalian gene (Hallböök et al., 1991; Ip et al., 1992), also known as NT-5 (Berkemeier et al., 1991), was subsequently isolated from rat and human DNA. NT-4/5 binds to the TrkB receptor, which also binds BDNF but at a different interaction site (Klein et al., 1992; Ip et al., 1993). NT-4/5 may also bind to p75, the low-affinity neurotrophin receptor (Hallböök et al., 1991).

In the present study, retrograde neuronal tracers and cell markers were used to study the response of injured RGCs in adult rat retinal explants to the administration of NT-4/5, a neurotrophin whose actions on these CNS neurons have not been previously investigated *in vitro*. The results of these experiments indicated that NT-4/5 was similar to BDNF in enhancing RGC survival and neurite outgrowth. Other growth factors (NGF, CNTF, and NT-3) appeared to have little effect on this specific population of retinal neurons.

The protective effects of BDNF on RGCs have been documented in embryonic retinas (Johnson et al., 1986), adult rat retinal explants (Thanos et al., 1989), and *in vivo* following optic nerve transection (Mansour-Robaey et al., 1994). In parallel with the present *in vitro* investigations, intravitreal administration of NT-4/5 *in vivo* temporarily pre-

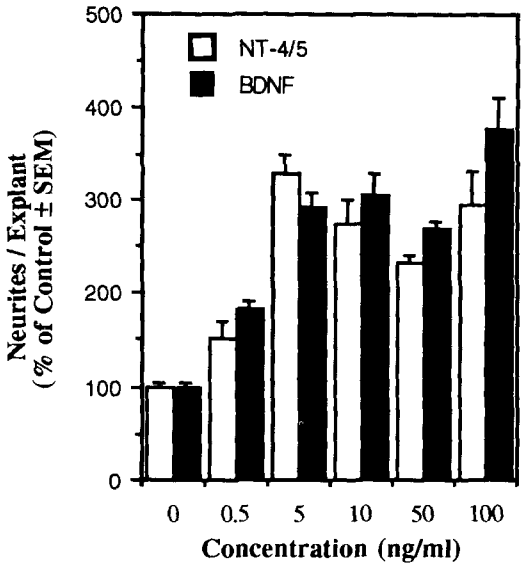

Figure 5 Dose–response effects of NT-4/5 and BDNF. The number of RT97 positive neurites extending from the retinal explant 7 days after treatment were calculated as a percentage of the control ± S.E.M. Maximal effect was observed at 5 ng/ml. All the values obtained in cultures treated with 5–100 ng/ml of NT-4/5 or BDNF were statistically significant ($p < 0.01$) when analyzed by ANOVA followed by a post-hoc Scheff's test.

vented the massive loss of RGCs caused by optic nerve transection (Clarke et al., 1993).

Consistent with the effects of NT-4/5 or BDNF *in vitro* and *in vivo*, we also showed by immunostaining that TrkB receptors are expressed on RGCs neurites extending from the adult rat retinal explants. Both NT-4/5 and BDNF bind and activate the TrkB receptor (Klein et al., 1992; Ip et al., 1993), which is expressed in RGCs *in vitro* and *in vivo* (Jelsma et al., 1993). The effects of NT-4/5 and BDNF were not additive, indicating that they probably share the same signaling pathway through activation of the TrkB receptor. The inability to demonstrate TrkA receptors on RGCs is consistent with the lack of NGF effect on these neurons described in the present studies.

In vivo studies of the effects of optic nerve transection demonstrated the delayed apoptotic death of RGCs (Berkelaar et al., 1994). We interpret the FG-labelled RGCs observed *in vitro* as an indication of the RGC population that survives in explants and attribute the greater numbers observed after the addition of NT-4/5 or BDNF to the direct effects of these neurotrophins on RGC survival (Mansour-Robaey et al., 1994; Clarke et al., 1993). In the present study, the neurotrophins presumably prevented or delayed the death of the RGCs axotomized at the time of the conditioning lesion or explantation.

The number of neurites per explant probably reflects RGC survival as well as growth, although we could not determine the extent to which NT-4/5 affected each of these two parameters. *In vitro*, the addition of NT-4/5 or BDNF caused greater increases in neurite formation than RGC survival (Fig. 4). While these discrepancies could be due to differences in the efficiency of RGC back-labelling and neurite immunolabelling, they could also be explained by increased branching and growth of axons. The observation that BDNF and NT-4/5 does indeed enhance the axonal growth and branching of axotomized RGCs *in vivo* (Mansour-Robaey et al., 1994; H. Sawai, G. M. Bray, and A. J. Aguayo, unpublished observations) provides

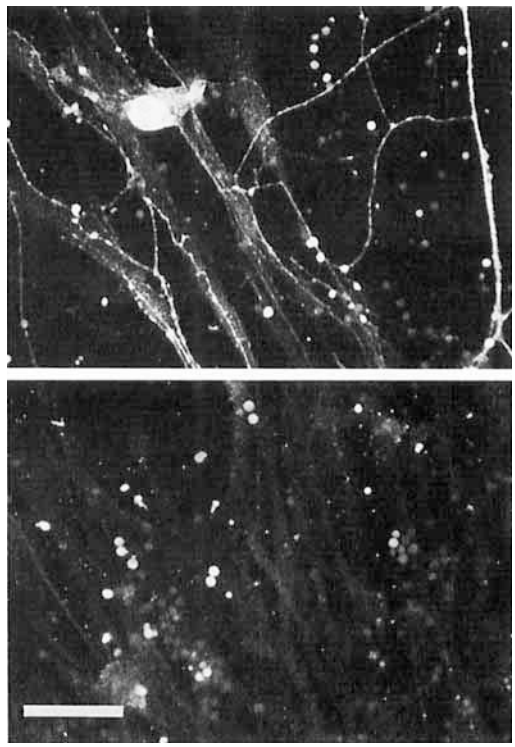

Figure 6 RGC neurites that extend from the explants express TrkB but not TrkA receptors. Explants treated with NT-4/5 were fixed after 7 days *in vitro* with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and incubated for 1 h at room temperature with rabbit antibodies to TrkB (upper) or TrkA (lower). After washing, the cultures were incubated with biotinylated goat anti-rabbit antibodies for 1 h, followed by fluorescein-avidin for 1 h. Neurites were immunopositive for TrkB but not for TrkA. Scale bar = 50 μm.

additional support to the significance of our documentation of RGC neurite extension *in vitro*.

The assistance of J. Trecarten, M. David, J. Laganière, and W. Wilcox is gratefully acknowledged. A.C. is a fellow of the Canadian Network on Neural Regeneration. This work was supported by grants from the Multiple Sclerosis Society of Canada and the Medical Research Council.

REFERENCES

ALLCUTT, D., BERRY, M., and SIEVERS, J. (1984). A quantitative comparison of the reactions of retinal ganglion cells to optic nerve crush in neonatal and adult mice. *Dev. Brain Res.* **15**:219–230.

ANDERTON, B. H., DOWNES, M. J., TOMLINSON, P. J., ULRICH, J., WOOD, J. N., and KAHN, J. (1982). Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature* **298**:84–86.

BÄHR, M., VANSELOW, J., and THANOS, S. (1988). *In vitro* regeneration of adult rat ganglion cell axons from retinal explants. *Exp. Brain Res.* **76**:393–401.

BARNSTABLE, C. J. and DRAGER, U. C. (1984). Thy-1 antigen: a ganglion cell specific marker in rodent retina. *Neuroscience* **11**:847–855.

BARRON, K. D., DENTINGER, G., KROHEL, G., EASTON, S. K., and MANKES, R. (1986). Qualitative and quantitative ultrastructural observations on retinal ganglion cell layer of rat after intraorbital optic nerve crush. *J. Neurocytol.* **15**:345–362.

BERKELAAR, M., CLARKE, D., WANG, Y.-C., BRAY, G. M., and AGUAYO, A. J. (1994). Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J. Neurosci.* **14**:4368–4374.

BERKEMEIER, L. R., WINSLOW, J. W., KAPLAN, D. R., NIKOLICS, K., GOEDDEL, D. V., and ROSENTHAL, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* **7**:857–866.

CLARKE, D. B., WANG, Y.-C., BRAY, G. M., RASMINSKY, M., and AGUAYO, A. J. (1993). Effect of Neurotrophin-4 on the survival of axotomized retinal ganglion cells in adult rats. *Soc. Neurosci. Abstr.* **19**:1104.

FORD-HOLEVINSKI, G., HOPKINS, J. M., MCCOY, J. P., and AGRANOFF, B. W. (1986). Laminin supports outgrowth from axotomized adult rat retinal axons. *Dev. Brain Res.* **28**:121–126.

GRAFSTEIN, B. and INGOGLIA, N. A. (1982). Intracranial transection of the optic nerve in adult mice: preliminary observations. *Exp. Neurol.* **76**:318–330.

HALLBÖÖK, F., IBAÑEZ, C. F., and PERSSON, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron* **6**:845–858.

IBÁÑEZ, C. F., ERNFORS, P., TIMMUSK, T., IP, N. Y., ARENAS, E., YANCOPOULOS, G. D., and PERSSON, H. (1993). Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development* **117**:1345–1353.

IP, N. Y., IBÁÑEZ, C. F., NYE, S. H., MCCLAIN, J., JONES, P. F., GIES, D. R., BELLUSCIO, L., LE BEAU, M. M., ESPINOSA, R., III, SQUINTO, S. P., PERSSON, H., and YANCOPOULOS, G. D. (1992). Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89**:3060–3064.

IP, N. Y., STITT, T. N., TAPLEY, P., KLEIN, R., GLASS, D. J., FANDL, J., GREENE, L. A., BARBACID, M., and YANCOPOULOS, G. D. (1993). Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron* **10**:137–149.

JELSMA, T. N., FRIEDMAN, H. H., BERKELAAR, M. J., BRAY, G. M., and AGUAYO, A. J. (1993). Different forms of the neurotrophin receptor trkB mRNA predominate in rat retina and optic nerve. *J. Neurobiol.* **24**:1207–1214.

JOHNSON, J. E., BARDE, Y.-A., SCHWAB, M., and THOENEN, H. (1986). Brain-derived neurotrophic factor supports the survival of cultured retinal ganglion cells. *J. Neurosci.* **6**:3031–3038.

KLEIN, R., LAMBALLE, F., BRYANT, S., and BARBACID, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8**:947–956.

MANSOUR-ROBAEY, S., CLARKE, D. B., WANG, Y.-C., BRAY, G. M., and AGUAYO, A. J. (1994). Effects of ocular injury and the administration of brain-derived neurotrophic factor (BDNF) on the survival and regrowth of axotomized retinal ganglion cells. *Proc. Natl. Acad. Sci. USA* **91**:1632–1636.

MISANTONE, L. J., GERSHENBAUM, M., and MURRAY, M. (1984). Viability of retinal ganglion cells after optic nerve crush in adult rats. *J. Neurocytol.* **13**:449–465.

THANOS, S., BÄHR, M., BARDE, Y.-A., and VANSELOW, J. (1989). Survival and axonal elongation of adult rat retinal ganglion cells. *In vitro* effects of lesioned sciatic nerve and brain derived neurotrophic factor. *Eur. J. Neurosci.* **1**:19–26.

VIDAL-SANZ, M., VILLEGAS-PÉREZ, P., BRAY, G. M., and AGUAYO, A. J. (1988). Persistent retrograde labeling of adult rat retinal ganglion cells with the carbocyanine dye dil. *Exp. Neurol.* **102**:92–101.

VILLEGAS-PÉREZ, M. P., VIDAL-SANZ, M., RASMINSKY, M., BRAY, G. M., and AGUAYO, A. J. (1993). Rapid and protracted phases of retinal ganglion cell loss follow axotomy in the optic nerve of adult rats. *J. Neurobiol.* **24**:23–36.

WONG, V., ARRIAGA, R., IP, N. Y., and LINDSAY, R. M. (1993). The neurotrophins BDNF, NT-3 and NT-4/5, but not NGF, up-regulate the cholinergic phenotype of developing motor neurons. *Eur. J. Neurosci.* **5**:466–474.