Enhanced Synthesis of Brain-derived Neurotrophic Factor in the Lesioned Peripheral Nerve: Different Mechanisms Are Responsible for the Regulation of BDNF and NGF mRNA

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Abstract. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are molecules which regulate the development and maintenance of specific functions in different populations of peripheral and central neurons, amongst them sensory neurons of neural crest and placode origin. Under physiological conditions NGF is synthesized by peripheral target tissues, whereas BDNF synthesis is highest in the CNS. This situation changes dramatically after lesion of peripheral nerves. As previously shown, there is a marked rapid increase in NGF mRNA in the nonneuronal cells of the damaged nerve. The prolonged elevation of NGF mRNA levels is related to the immigration of activated macrophages, interleukin-1 being the most essential mediator of this effect.

Here we show that transsection of the rat sciatic nerve also leads to a very marked increase in BDNF mRNA, the final levels being even ten times higher than those of NGF mRNA. However, the time-course and spatial pattern of BDNF mRNA expression are distinctly different. There is a continuous slow increase of BDNF mRNA starting after day 3 post-lesion and reaching maximal levels 3–4 wk later. These distinct differences suggest different mechanisms of regulation of NGF and BDNF synthesis in non-neuronal cells of the nerve. This was substantiated by the demonstration of differential regulation of these mRNAs in organ culture of rat sciatic nerve and Schwann cell culture. Furthermore, using bioassays and specific antibodies we showed that cultured Schwann cells are a rich source of BDNF- and ciliary neurotrophic factor (CNTF)-like neurotrophic activity in addition to NGF. Antisera raised against a BDNF-peptide demonstrated BDNF-immunoreactivity in pure cultured Schwann cells, but not in fibroblasts derived from sciatic nerve.

BRAIN-derived neurotrophic factor (BDNF)[1] and nerve growth factor (NGF) belong to a still growing family of neurotrophic molecules collectively called neurotrophins (6, 60). These proteins show a similar basic structure reflected by conserved domains arranged around the six cysteine residues which seem to be of great importance in determining the three-dimensional structure of these molecules, a prerequisite for their biological activity. However, BDNF and NGF also show distinctly different variable domains which determine the different spectra of their neuronal specificity. Both BDNF and NGF support neural crest–derived sensory neurons in the periphery (placode-derived sensory neurons are supported only by BDNF and neurotrophin-3 [NT-3]) (27, 28, 37). The effects of different neurotrophins on DRG neurons are additive in vitro (BDNF and NGF, see reference 37; BDNF and NT-3, see reference 28), suggesting that the various sub-populations of neurons are supported by different members of the NGF gene family in a partly overlapping manner. Under physiological as well as culture conditions NGF is produced in target tissues of mixed peripheral nerves, such as skin and iris (4, 7, 13, 14, 20, 21, 54, 55), whereas BDNF expression has not been detected in the periphery, with the exceptions of lung, heart, and skeletal muscle (26, 33, 40) where the cellular site of synthesis remains to be established. However, in the central nervous system (CNS) expression of BDNF mRNA is more extensive including the spinal cord (26, 40, 68). Therefore, it seems reasonable to assume that DRG sensory neurons obtain BDNF via their central processes. BDNF mRNA has recently also been detected in DRG neurons (15, 16). It remains unclear whether conditions exist where these neurons are supplied with BDNF in an autocrine or paracrine manner.

NGF is not expressed to a significant extent in non-neuronal cells of adult peripheral nerves (22). However, this situation changes dramatically after nerve lesion. A rapid, transient, and massive increase in NGF mRNA occurs in the nerve region immediately proximal to the lesion and all

1. *Abbreviations used in this paper*: BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion; FK, forskolin; IL-1, interleukin-1; NGF, nerve growth factor; NT-3, neurotrophin-3.

along the distal nerve segment. This rapid increase is followed by a second increase in NGF mRNA which lasts several weeks. The second long-lasting increase is mostly due to IL-1 released by macrophages invading the lesioned nerve. This can be deduced from the observation that the second increase of NGF mRNA in vivo coincides with the invasion of macrophages at the lesion site (23). An initial rapid increase without the subsequent second phase is observed in nerve organ culture. However, the second increase of NGF mRNA observed in vivo can be mimicked by the addition of activated macrophages or even recombinant IL-1 to organ cultures (23, 36), and can be blocked by IL-1 antibodies (36). The role played by macrophage-secreted IL-1 is further supported by the recent observation that the mouse strain Ola (which is defective in macrophage invasion into lesioned nerves) only shows the rapid immediate increase and a much smaller secondary increase in NGF mRNA (9). This smaller increase is paralleled by the lower increase in IL-1β mRNA corresponding to the highly reduced invasion of macrophages (24). We have now analyzed the expression of BDNF mRNA, BDNF-like activity and the localization of BDNF-like immunoreactivity in the transsected peripheral rat nerve and in isolated Schwann cells and fibroblasts of the rat sciatic nerve. As previously shown for NGF, BDNF mRNA increases to high levels after lesion. However, the time-course of the elevation of BDNF mRNA levels is distinctly different from that of NGF mRNA. In contrast to NGF mRNA, the increase in BDNF mRNA distal to the transsection site is monophasic and takes place at a much slower rate, reaching maximal levels at approximately four weeks posttranssection. The maximal BDNF mRNA levels are about 10 times higher than those of NGF mRNA. These observations demonstate that different regulatory mechanisms control the levels of BDNF and NGF mRNA in the lesioned nerve in vivo. Specifically, IL-1 does not alter BDNF mRNA levels in explant cultures of sciatic nerve. Moreover, in vitro synthesis of BDNF is restricted to Schwann cells and the levels of BDNF and NGF mRNA in these cells are differentially regulated.

The massive increase in BDNF mRNA in sciatic nerve and in isolated Schwann cells is of particular interest in view of previous observations that peripheral nerve grafts and Schwann cells proved to be conducive for the regeneration of central neurons, in particular retinal ganglion cells (31, 39, 59, 64) and dopaminergic striatal neurons (11, 12, 17, 30, 62) which are supported by BDNF, but not by NGF.

Materials and Methods

Materials

Forskolin (7β-acetoxy-8,13-epoxy-1a,6β,9α-trihydroxylabd-14-ene-11-one); ionomycin; phorbol 12-myristate 13-acetate (TPA); 8-bromo-cAMP; and N^6-benzoyl-cAMP were purchased from Sigma Chemical Co. (Deisenhofen, Germany). Purified porcine TGFβ1 was obtained from British Bio-Technology (Oxford, England). Human recombinant IL-1β was a generous gift of Drs. A. Gronenborn (Max-Planck-Institute for Biochemistry, Martinsried, Germany) and P. Wingfield (Biogen/Glaxo, Geneva, Switzerland).

Recombinant Proteins and Antibodies

Recombinant mouse NT-3 was produced and purified as described by Götz et al. (1992). Recombinant mouse BDNF was produced by the same method. They were generously provided by Y.-A. Barde and R. Kolbeck (Max-Planck-Institute for Psychiatry, Department of Neurochemistry, Martinsried, Germany). The biological activity of recombinant BDNF was identical to that of BDNF purified from pig brain (27). BDNF-peptide specific antisera were produced and characterized as previously described by Wetmore et al. (1991). Briefly, polyclonal antisera have been raised against a synthetic peptide derived from $BDNF_{173-182}$. Recombinant rat CNTF was a kind gift of M. Sendtner (Max-Planck-Institute for Psychiatry, Department of Neurochemistry, Martinsried, Germany) and was produced as described by Masiakowski et al. (1991). The CNTF antiserum was kindly provided by M. Sendtner. It was raised by immunization of rabbits with recombinant rat CNTF. An IgG fraction was purified on a protein G column (Pharmacia Fine Chemicals, Freiburg, Germany) according to the manufacturer's instructions, dialyzed extensively, lyophilized, and used at the indicated concentrations.

Surgical Procedures

Wistar rats (180–250 g) were ether anaesthetized and a 7–10-mm piece of the right sciatic nerve was resected with minimal damage of surrounding tissues. No further attempts were made, such as ligation or deflection of the proximal stump, to prevent reinnervation of the distal stump. Clips were used to close the wound. After indicated times rats were killed by cervical dislocation and the nerves dissected according to the scheme presented in Fig. 1 *B* and rapidly frozen in liquid nitrogen.

Cell Culture

Pieces of adult and newborn rat sciatic nerve (six pieces of 5-mm length) were kept in culture (10% FCS, DME) for 3 d with daily changes of media as previously described (23, 44). The indicated treatments were started the third day.

Schwann cell cultures were performed as previously described in detail by Matsuoka et al. (1991*b*). During selection and expansion of cultures cytosine-arabinoside and forskolin (FK) were used. For a discussion of possible influences of these drugs of Schwann cell phenotype and NGF mRNA expression see Matsuoka et al. (1991b).

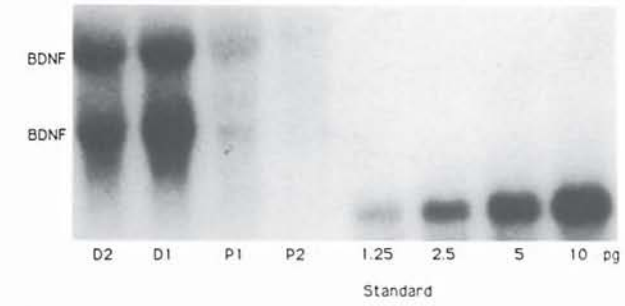
The dissociation and tissue digestion methods used for Schwann cell cultures have also been used for fibroblast cultures derived from nerves of 1–3-d-old rats. Cytosine-arabinoside selection was however omitted. Such cultures normally develop into cultures heavily contaminated by Schwann cells situated above a fibroblast monolayer. Schwann cells were eliminated from these cultures by treatment with 10 μM FK starting the second day in vitro. Consistent with previous observations Schwann cells need, in addition to FK and growth factors contained in FCS, a specific substrate, in this case poly-lysine, to proliferate (47, 65). However, fibroblasts from the same nerves grow in FK-containing media on plain tissue culture plastic. Seven to nine days after initiation of the FK treatment confluent monolayers of fibroblasts (characterized by indirect immunofluorescence as described by Matsuoka et al., 1991*b*) (>90% Thy 1.1 positive [OX7 monoclonal], <10% S100 positive) were obtained. These were passaged once without FK, grown to confluency and used for experiments after at least one additional medium change. Thus, they were kept without FK for at least 8 d.

Northern Blot

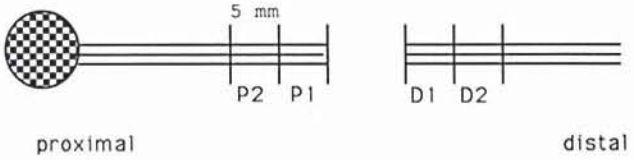
Northern blot analysis of mRNA was performed as previously described in detail (20, 44). Briefly, total RNA was isolated from cultured cells and homogenized tissue by the acid phenol method (10), denatured, and separated on agarose gels, vacuum-blotted to nylon and probed with a NGF cRNA probe (44), a BDNF cRNA probe (26) or a Po cDNA probe (34). To allow for correction of different extraction efficiencies a 0.5-kb synthetic NGF-RNA or BDNF-RNA were included during extraction. Autoradiograms were scanned by laser densitometry. Absolute amounts of specific mRNA were calculated from standards analyzed simultaneously on each gel. Values were corrected for differences in extraction efficiencies and length of transcripts. The absolute amounts of BDNF mRNA are given for the smaller of the two transcripts.

Bioassays

The bioassay on dissociated E8 chick ciliary neurons has been described previously (29). Initially plated neurons and neurons surviving in the presence of samples after 24 h were counted. Results were calculated as given in the table legend. Samples tested in this bioassay were cell culture media conditioned for 3 d on a confluent layer of Schwann cells cultured as described above. At the time of conditioning these cells had received at least three medium changes to FK-free medium. Thus, they had been kept for at least 10 d without FK. Conditioned media were centrifuged (400 *g*) to



A



B

Figure 1. (*A*) BDNF mRNA in different segments of the transsected sciatic nerve 17 d after lesion. *P*, proximal segments; *D*, distal segments. Labeling of segments is indicated in *B*. (*B*) Dissection scheme.

remove cellular debris and frozen until use. Final dilutions of conditioned media in the bioassay are indicated in the table legends. Wells used to assay recombinant proteins and controls received an equal amount of fresh DME, 10% FCS which was also used for Schwann cell cultures.

The bioassay on dissociated chick E8 nodose ganglion neurons was performed as previously described (37). Results were obtained after 72 h of culture as described in the table legend. Conditioned media were prepared as detailed for the ciliary neuron bioassay but without dilution.

Immunofluorescence of Cultured Cells

Cultures used for staining were either expanded cultures as described above, mixed cultures obtained after culturing for 2 d without cytosine-arabinoside selection or mixed cultures obtained by co-culture of purified expanded populations of Schwann cells and sciatic fibroblasts (see above). Cells were passaged to poly-lysine–coated glass coverslips and cultured for 1–3 d before fixation (15 min) at room temperature in 4% PFA, 0.1% picric acid. Incubations with antisera serving as first antibodies were performed overnight at the indicated dilutions as described (67). To reduce unspecific staining 5% normal horse serum was added to the primary antiserum incubations. Primary antibodies were detected using a biotinylated anti-rabbit antibody (1:100) (Amersham Corp., Arlington Heights, IL) and a streptavidin-FITC or streptavidin-Texas Red conjugate (1:200) (Amersham Corp.). Epifluorescence microscopy was performed on a Zeiss axiophot microscope (Carl Zeiss, Oberkochen, Germany). Antisera from different rabbits and a protein-A–purified IgG fraction of one of them have been used for the staining experiments.

Results

Effect of Sciatic Nerve Transsection on BDNF mRNA Levels in the Nerve Sheath

In a first series of experiments we analyzed the expression of BDNF mRNA in intact and transsected sciatic nerves of adult rats. A 7–10-mm piece of nerve was resected unilaterally and two segments of 5-mm length proximal and distal to the lesion were used for the quantification of BDNF mRNA levels at the indicated times after transsection (Fig. 1 *B*). Comparisons were made on a wet weight basis. Basal levels are below the detection limit (16 fg/mg wet weight).

Clearly elevated levels of 210 fg/mg wet weight were first detected 7 d post-lesion distal to the transsection site (Fig. 2). This increase in BDNF mRNA level continued until 620 fg/mg was detected 21 d post-lesion. A much smaller increase was observed between day 21–28. In no case were fiber strands bridging the gap between the distal and proximal stumps observed during dissection. As a control for possible regeneration, blots were reprobed with a rat Po cDNA probe. It is well established that Po mRNA is downregulated in distal parts of transsected peripheral nerves and stays at a low level as long as regenerating fibers do not reach the distal Schwann cell bands (61). All nerves analyzed more

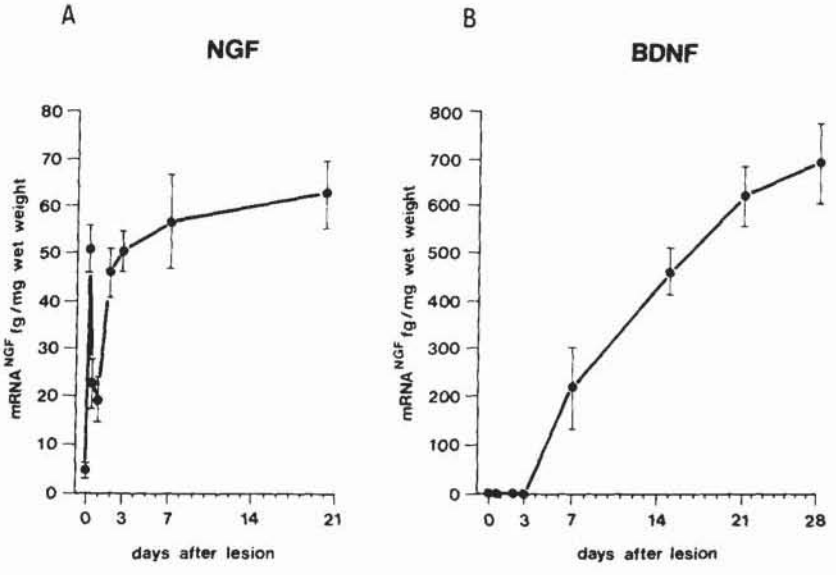


Figure 2. (*A*) For comparison, the time course of NGF mRNA changes in the first distal segment of the transsected rat sciatic nerve is shown (taken from reference 17). (*B*) Time course of BDNF mRNA changes in the first distal segment D1 after resection as described in Materials and Methods. Levels of BDNF mRNA at day 1 through day 3 after lesion were below the detection limit (16 fg/mg wet weight). Average and SD of three independent experiments are shown.

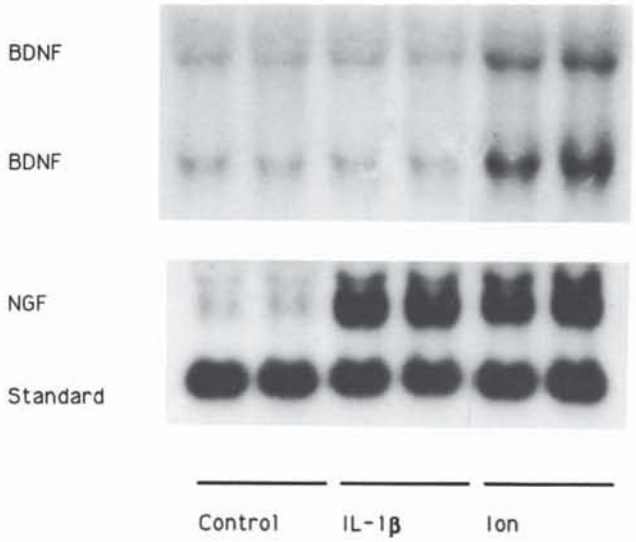


Figure 3. Comparison of BDNF mRNA and NGF mRNA in newborn nerve organ cultures. (*Top*) BDNF mRNA; (*bottom*) NGF mRNA. (*Standard*) NGF recovery standard. (*IL-1B*) 50 U/ml, 3 h; (*Ion*) ionomycin, 1 μg/ml, 3 h. For each treatment duplicate samples are shown.

than 14 d after lesion did not contain detectable Po mRNA in distal segments (data not shown).

Interestingly, the elevation of BDNF mRNA levels was restricted to distal segments and was most pronounced in the segment immediately distal to the transsection site (Fig. 1 *A*).

As described for other tissues, BDNF mRNA was detected as two bands of 1.5 to 1.7 kb and ~4.6 kb. No additional bands were seen. The molar ratio of the small to long transcript was ~3 at all times after lesion (Fig. 1 *A*). This ratio is similar to the one described for other tissues (26, 40).

Regulation of BDNF mRNA Levels in Nerve Organ Culture

Since the maximal increase in BDNF mRNA in vivo was not reached before 2 wk post-lesion a regulatory role for secreted macrophage products is unlikely. As described earlier, administration of IL-1β (which is a major product released by activated macrophages) to nerve organ cultures derived from newborn or adult animals resulted in a marked increase in NGF mRNA (36, 44). However, this was not accompanied by any change in BDNF mRNA levels (Fig. 3). Similar results were obtained in adult nerve organ cultures

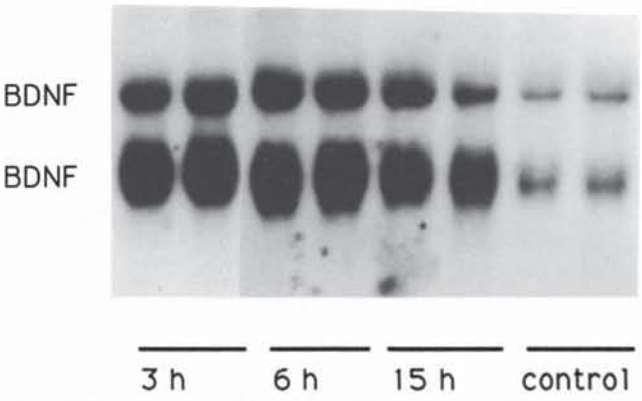


Figure 5. Time course of the effect of ionomycin on BDNF mRNA levels in cultured Schwann cells. Ionomycin was given at 1 μg/ml for the indicated times. Duplicate samples are shown.

(data not shown). Basal levels of BDNF mRNA in these culture systems were clearly detectable and higher than levels in intact nerve in vivo. This effect of culturing has also been previously observed for NGF mRNA levels. However, BDNF mRNA levels in organ culture were not maximal as they could be further increased by ionomycin treatment (Fig. 3). This is in agreement with the data obtained in Schwann cell cultures (see below).

Regulation of BDNF mRNA in Cultured Schwann Cells by Polypeptides and Second Messenger Pathways

The analysis was extended to Schwann cells as the most abundant non-neuronal cell type of the sciatic nerve. As reported previously, cultured Schwann cells prepared from newborn sciatic nerve constitutively express BDNF mRNA (1, 43) (Fig. 4, lanes *1* and *2*, *control*). This basal expression amounted to ~18 pg/10^6 cells. Thus cultured Schwann cells contain clearly elevated amounts of this mRNA compared to BDNF mRNA levels in intact nerve. Administration of ionomycin resulted in a rapid increase of BDNF mRNA levels (two- to threefold) similar to that found in organ cultures (Fig. 5 and Table I). The effect of ionomycin could be further potentiated by co-administration of TPA (~10-fold) (Table I). TPA alone had a small suppressive effect on BDNF mRNA levels (Table I).

Besides the two mRNA transcripts referred to above no additional band was seen. The ratio of small to long mRNA was, again, ~3.

In agreement with the results reported for nerve organ culture no effect of IL-1 was observed (Table I).

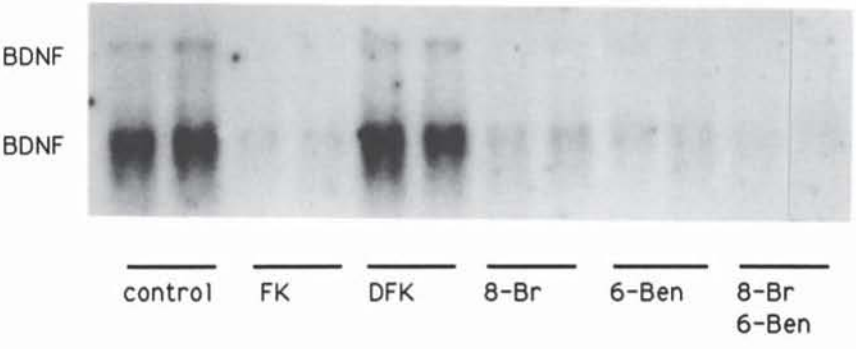


Figure 4. Regulation of BDNF mRNA in cultured Schwann cells by cAMP. (*FK*) forskolin, 10 μM; (*DFK*) dideoxyforskolin, 10 μM; (*8-Br*) 8-bromo cAMP, 0.5 mM; (*6-Ben*) N6-benzoyl-cAMP, 0.5 mM; (*8-Br*) 6-Ben: combination of both, each 0.5 mM. All treatments were done for 3 h. Duplicate samples are shown.

Table I. Regulation of Schwann Cell BDNF mRNA Levels by Growth Factors and Signal Transduction Pathways

Factor added	BDNF mRNA relative to control
Control	1
FK	0.33 ± 0.06
TPA	0.7 ± 0.17
IONO	2.7 ± 0.36
IONO + FK	0.75 ± 0.21
IONO + TPA	9.7 ± 1.9
IL-1β	0.9 ± 0.21
TGF-β1	0.18 ± 0.02

BDNF mRNA levels of cultured Schwann cells were analyzed by quantitative Northern blotting and values (average ± SD of three independent determinations) are given relative to control levels which were set 1. Factors used: (*FK*) 10 μM, 3 h; (*TPA*) 100 ng/ml, 3 h; (*IONO*) ionomycin, 1 μg/ml, 3 h; for combinations the same concentrations were applied for 3 h; (*TGF-b1*) 1 ng/ml, 24 h; (*IL-1β*) recombinant human IL-1β, 50 U/ml, 3 h.

Interestingly, FK which is a direct activator of adenylate cyclase and induces NGF mRNA in Schwann cells, reduced BDNF mRNA levels reproducibly to 33% of basal levels (Fig. 4). Dideoxyforskolin, an analogue of FK which does not activate adenylate cyclase, failed to reduce BDNF mRNA. Furthermore, membrane-permeable derivatives of cAMP also elicited a reduction of BDNF mRNA levels comparable to that seen with FK (Fig. 4). The effect of cAMP elevation by FK could not be suppressed by an inhibitor of protein kinase C (H7, 50 μM) nor, remarkably, by an inhibitor of protein kinase A (H8, 50 μM) (data not shown) (25). Forskolin treatment also suppressed the increase of BDNF mRNA levels induced by ionomycin (Table I). Treatment of Schwann cells with TGF-β1 which has previously been shown to reduce basal NGF mRNA expression (44) also resulted in a clear reduction of BDNF mRNA levels (Table I).

Fibroblast cultures purified and expanded as described in Materials and Methods contained ~10% of the BDNF mRNA levels determined for Schwann cell cultures when equal amounts of total RNA were analyzed (data not shown).

Neurotrophic Activity in Schwann Cell–conditioned Media

This series of experiments was performed with the goal to establish whether the BDNF mRNA levels in Schwann cells are reflected by a corresponding production of BDNF-like activity. Since neither a specific immunoassay nor blocking antibodies for BDNF are available, the determination of BDNF-like activity depended on an appropriate bioassay.

To evaluate the presence of cross-reacting neurotrophic activity we initially tested Schwann cell conditioned media in an assay using ciliary ganglion neurons. These neurons do not respond to BDNF or to NGF (37; see reference 52 and data not shown). This assay was chosen to investigate the presence of CNTF, which has a potent neurotrophic activity on a large variety of neurons (see reference 52) and thus might interfere with other bioassays. Schwann cell–conditioned media contained enough CNTF-like activity to rescue ~50% of the plated neurons at a 1/10 dilution (Table II). A polyclonal antiserum produced against recombinant rat CNTF, which completely blocks the effect of 1 ng/ml of recombinant CNTF, reduced the survival activity of Schwann cell–conditioned media to 15%.

Dissociated nodose ganglion neurons of E8 chick were used as a BDNF-sensitive bioassay. In confirmation of previous results (52), recombinant CNTF has a substantial survival effect in this assay which can however be blocked by CNTF-antiserum (data not shown). Thus anti-CNTF antiserum was added to all samples. Native undiluted conditioned media showed high survival activity in this assay, rescuing 86% of all plated neurons (Table III). Inclusion of anti-CNTF resulted in a reduction to 53% surviving neurons. Due to the unavailability of neutralizing antisera against BDNF it was necessary to use an indirect way to demonstrate that the remaining activity, at least in part, represented BDNF-like activity. If Schwann cell–conditioned media were to contain saturating concentrations of BDNF, one would predict no increase in survival activity by addition of recombinant BDNF. Addition of a saturating dose of BDNF to anti-CNTF treated samples resulted in only a small increase of neuronal survival (Table III). The same concentration of exogenous BDNF alone in the presence of anti-CNTF rescued 37% of the plated neurons. Thus the lack of a distinct additional effect of exogenous BDNF suggests that Schwann

Table II. Neurotrophic Activity of Schwann Cell–conditioned Media: Bioassay on E8 Chick Ciliary Ganglion Neurons

Factors added	Percent specific survival
SC-CM	54 ± 12.9
SC-CM + anti-CNTF, 5 μg/ml	19*
SC-CM + anti-CNTF, 10 μg/ml	13*
SC-CM + anti-CNTF, 20 μg/ml	15 ± 2.5
SC-CM + anti-CNTF, 40 μg/ml	11*
CNTF	93 ± 12.7
SC-CM + CNTF	85‡

The assay was performed on dissociated E8 chick ciliary ganglion neurons. Surviving cells were counted after 48 h and percentages were calculated as for Table III. Background survival was 0% in all experiments. If not indicated otherwise average ± SD were calculated from three independent experiments assaying three different Schwann cell–conditioned media.
(*SC-CM*) Obtained as given in Materials and Methods, used at a final dilution of 1/10; (*CNTF*) recombinant rat CNTF, 1 ng/ml; (anti-CNTF) neutralizing CNTF antiserum as described.
* Single value.
‡ Average of two independent experiments using two different SC-CM.

Table III. Neurotrophic Activity of Schwann Cell–conditioned Media: Bioassay on E8 Nodose Neurons

Factors added	Percent specific survival
SC-CM	86 ± 5.7
SC-CM + anti-CNTF	53 ± 3.6
SC-CM + anti-CNTF + BDNF	60 ± 5.7
anti-CNTF + BDNF	37 ± 9.5
SC-CM + anti-CNTF + NT-3	70 ± 3.2
anti-CNTF + NT-3	34 ± 6.4
anti-CNTF + BDNF + NT-3	63 ± 4.9

The assay was performed on dissociated E8 chick nodose ganglion neurons. Surviving cells after 72 h in culture were calculated as a percentage of initially plated cells. Survival without added factors has been subtracted (background values for the three experiments done were: 1, 10, 2%). Averages ± SD of three independent experiments assaying three different samples of Schwann cell conditioned media are given. Factors added: (SC-CM) Undiluted Schwann cell conditioned medium, containing 10% FCS, conditioned for 3 d; (*anti-CNTF*) IgG-fraction of a neutralizing CNTF antiserum at 10 μg/ml; (*BDNF*) recombinant mouse BDNF, 1 ng/ml; (*NT-3*) recombinant mouse NT-3, 1 ng/ml. Factors were added once and were present throughout the whole culture period.

cell-conditioned media contained concentrations of BDNF-like activity which were close to saturation.

Addition of a saturating concentration of recombinant NT-3 to anti-CNTF treated conditioned medium samples resulted in higher increases of survival activity (Table III). This percentage is lower than the one achieved by addition of NT-3 alone. This and the fact that Schwann cell–conditioned media in the presence of neutralizing CNTF antibodies rescue 16% more neurons than BDNF alone suggests that these media contain a factor acting on NT-3 responsive neurons. The molecular identity of this neurotrophic activity has not been elucidated. It cannot be excluded that it, at least partly, represents NT-3, although preliminary Northern blotting experiments did not reveal the presence of NT-3 mRNA in cultured Schwann cells (levels were lower than ~250 fg/10^6 cells) (data not shown). For comparison, Schwann cells cultured as described here contain ~5.6 pg of NGF mRNA/10^6 cells (44) and ~18 pg of BDNF mRNA/10^6 cells (see above).

Intracellular Localization of BDNF-like Immunoreactivity

In an attempt to provide more direct evidence for the presence of BDNF protein in Schwann cells we used antisera raised against a peptide sequence contained in BDNF for immunostaining studies of cultured Schwann cells. These antisera have previously been extensively characterized by Wetmore et al. (1991). As our Northern blot analysis of fibroblasts derived from newborn sciatic nerves showed only very little specific BDNF mRNA, we used mixed cultures of Schwann cells and fibroblasts to provide an internal control of staining specificity.

These experiments revealed clear and evenly distributed staining of Schwann cells (Fig. 6 *A*). Most of the flat large cells present in the cultures were identified as Thy1 positive and S100 negative in separate immunostainings and thus probably represented fibroblasts. In most (~90%) of these cells no staining could be detected. However, ~10% of the flat cells showed a staining comparable in intensity to the staining of Schwann cells (Fig. 7 *A*). Closer examination of the staining revealed an exclusively extranuclear signal in both positive cell types (Figs. 6 *A* and 7 *A*). In many cells the most intensive staining was restricted to the perinuclear area (Fig. 6 *A*). However, a faint but clear signal was also detected in Schwann cell processes. At higher magnification the staining in Schwann cells appeared granular (Fig. 6 *C*). Antisera obtained from two different rabbits, and an IgG fraction purified from one of them, gave similar results.

Discussion

Comparison of the Expression Pattern of BDNF mRNA and NGF mRNA after Nerve Lesion

The data presented provide evidence that synthesis of BDNF is upregulated after peripheral nerve lesion in non-neuronal cells of the nerve sheath. A similar augmentation of synthesis has previously been reported for NGF (22). However, there are striking differences between the two neurotrophins regarding the time course, the magnitude of the response and its localization. The biphasic increase in NGF mRNA is characterized by a first very rapid and transient increase peaking at ~6 h after lesion and a second prolonged one starting around 2 to 3 d after lesion (22). In contrast, there is no detectable increase in BDNF mRNA for up to three days after lesion. Thereafter, amounts of BDNF mRNA rose slowly and reached maximal levels after 2 wk after lesion. There is good evidence that macrophage-derived IL-1 is responsible for the second phase of the NGF mRNA increase. Since a maximum of macrophage invasion and activation at the lesion site occurs between day 2 and 6 after transsection (8, 46), products of stimulated macrophages are not likely to play a role in the delayed and prolonged increase of BDNF mRNA. This notion is supported by the lack of effect of IL-1 on BDNF mRNA levels in nerve organ cultures which are a reasonable model for IL-I–mediated NGF mRNA regulation (23).

Interestingly, high levels of BDNF mRNA are found almost exclusively in distal nerve pieces. In contrast, a significant increase in NGF mRNA has been detected in the nerve stump immediately proximal to the lesion site which is also invaded by macrophages. Therefore this difference in the localization of trophic factor again might be explained by macrophage-independent BDNF induction.

Additionally, a biphasic production of neurotrophic activity has been previously described for rat DRG neurons by Windebank and Poduslo (1986). Distal nerve pieces dissected 3 to 7 d after transsection produce an activity which can be partly blocked by antisera to NGF. A second phase of production of neurotrophic activity which could not be blocked by these antisera was observed in pieces undergoing degeneration for 2 to 4 wk. This observation might be explained by the initial rapid release and production of NGF (22) and CNTF (41, 53, 69) and the delayed production of BDNF described here. It is unlikely that CNTF is responsible for the second phase since nerve lesion leads to a rapid decrease in CNTF mRNA and, with some delay, CNTF protein (53). Nerve pieces distal to a crush lesion or transsection have also been shown to produce survival activity for retinal ganglion cells (59). Retinal ganglion cells of adult rats after optic nerve lesion (59) and of embryonic rats (31) in culture can be kept alive by BDNF. The activity in sciatic exudate was not additive to BDNF suggesting that it was identical to BDNF (59). CNTF does not affect the survival of cultured embryonic retinal ganglion cells (31).

Under normal conditions both NGF and BDNF mRNA are barely detectable in intact sciatic nerve. However, the levels of both mRNAs are up-regulated after nerve lesion. The maximal tenfold higher levels of BDNF mRNA as compared to NGF mRNA indicate that cells of the nerve sheath under the appropriate circumstances can be a very rich source of BDNF. This is also interesting in view of the fact that BDNF has been regarded primarily as a centrally produced neurotrophin and that expression of BDNF mRNA outside the CNS has initially been detected in only two peripheral organs (lung and heart) (26, 33, 40) and later also in a subpopulation of DRG neurons (15, 16). BDNF mRNA has not been detected in adult mouse skin (40). Recently, however, constitutive levels of BDNF mRNA have been described in poly-A^+ RNA of newborn rat skin (1). The reasons for this discrepancy are unclear. Species and age differences, as well as the amount of RNA analyzed (~ eightfold more in the study by Acheson et al., 1991), may be responsible.

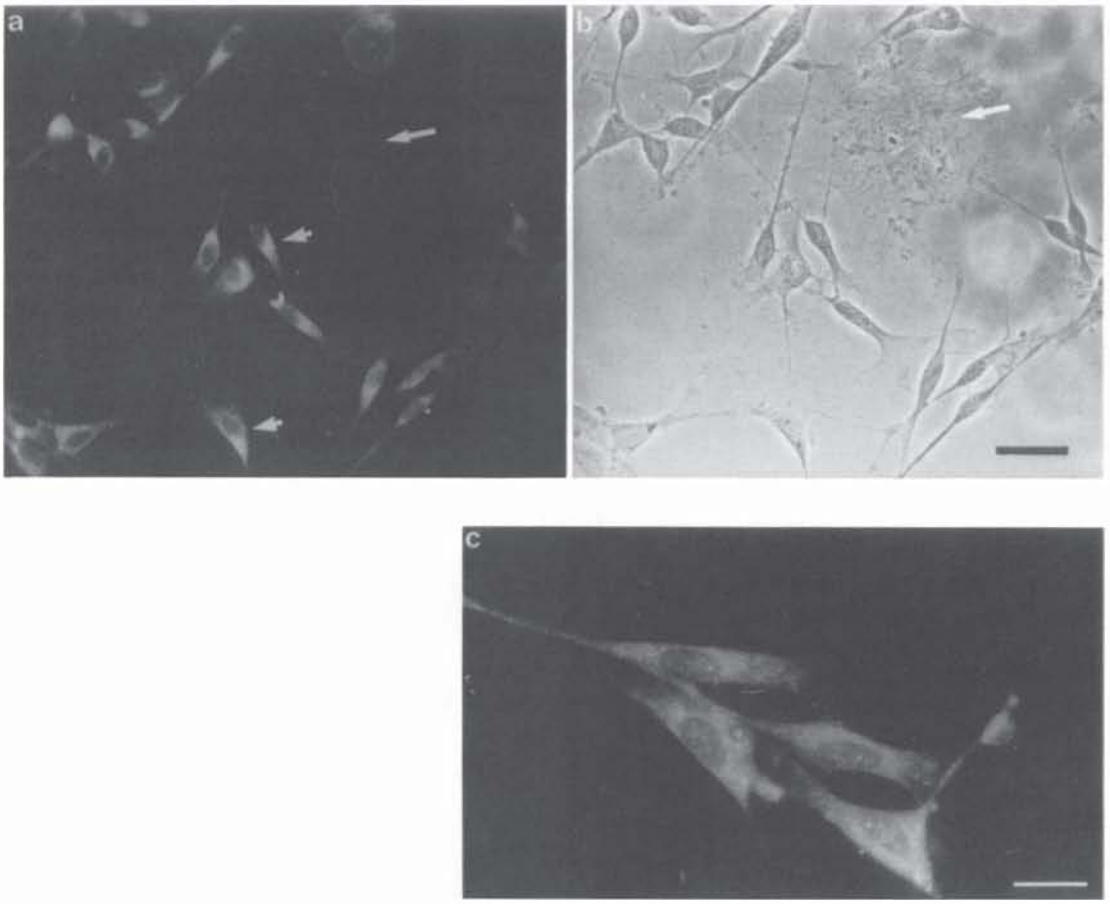


Figure 6. Immunolocalization of BDNF in primary mixed cultures of Schwann cells and fibroblasts after 2 d in vitro. (*a*, epifluorescence) Schwann cells (*small arrows*) show mainly perinuclear staining. Nuclei are spared. (*b*, phase contrast) One fibroblast (*long arrow*) is not stained in *a*. (*c*, epifluorescence) At higher magnification staining of Schwann cells appears as diffuse granules extending into cell processes. Whole antiserum diluted 1:500 was used. Bars: (*a* and *b*) 50 μm; (*c*) 25 μm.

Regulation of BDNF mRNA and NGF mRNA in Cultured Schwann Cells

Our data clearly demonstrate a differential regulation of the levels of BDNF mRNA and NGF mRNA in cultured Schwann cells and nerve organ cultures. In contrast to the six- to ten-fold increase in NGF mRNA levels induced by elevation of intracellular cAMP (44), a significant reduction of basal as well as ionomycin induced levels of BDNF mRNA is observed in cultured Schwann cells. This finding, together with the differential regulation observed in organ culture and the differences in the time course and localization of expression after lesion, suggests that fundamentally different mechanisms regulate the expression of these structurally related proteins. It is, however, not clear whether a situation analogous to cAMP elevation in culture occurs in vivo during degeneration/regeneration or development and what the extracellular signals mediating such changes might be. Stimulation of another set of kinases (particularly protein kinase C) by TPA had a weak suppressive effect on BDNF mRNA levels. A stronger suppressive effect has previously been observed on NGF mRNA levels (44). TGF-β1 exerts a strong suppressive effect on basal as well as stimulated NGF mRNA levels (44) and a similar effect on BDNF mRNA expression. Whether kinases activated by phorbol esters or signal transduction pathways activated by TGF-β1 might play a role in down-regulation of neurotrophin expression after regeneration, or in the maintenance of low neurotrophin levels in intact nerves deserves further investigation.

Release of Neurotrophic Activities by Cultured Schwann Cells

An important question is whether Schwann cells produce and release active BDNF protein. The tools available to study this question are still limited. With the discovery of additional neurotrophins acting on partly overlapping sets of target neurons, it is difficult to assure monospecificity of any bioassay used. Furthermore, neutralizing antibodies against BDNF are not yet available. We chose to assay survival of E8 chick nodose ganglion neurons since these neurons are responsive to BDNF, which may be released from Schwann cells after lesion, and are not responsive to NGF which is also present in Schwann cell–conditioned media (3, 44, 49). This bioassay does not distinguish between BDNF and NT-3, which support survival of these neurons in an additive fashion. Additionally, E8 chick nodose neurons also respond to CNTF (52) which is known to be expressed in sciatic nerve and in Schwann cells in vivo as well as in vitro (35, 48, 53, 56, 57). Therefore we quantified CNTF-like bioactivity in Schwann cell–conditioned media. We were surprised to

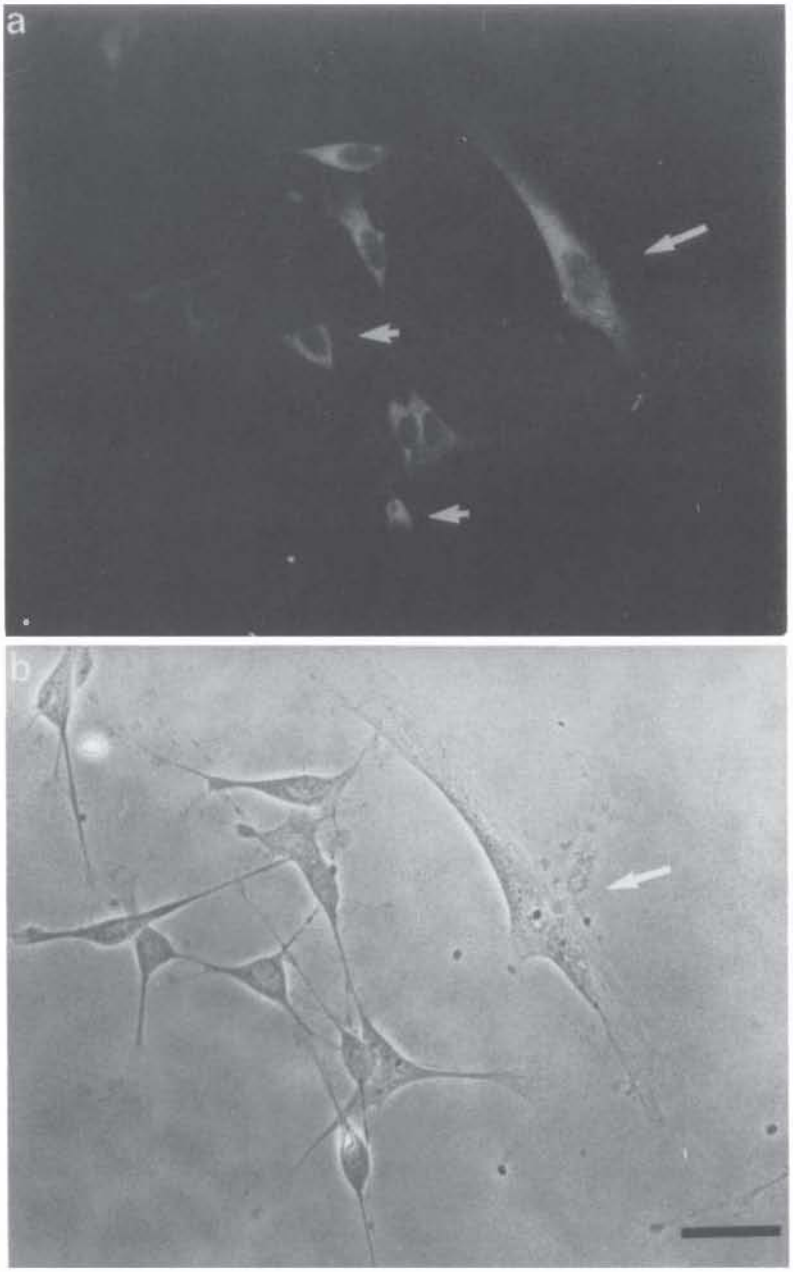


Figure 7. Immunolocalization of BDNF in primary mixed cultures after 2 d in vitro. (*a*, epifluorescence) All Schwann cells (*small arrows*) are stained mainly in the perinuclear region. Additionally, a flat long cell (*long arrow*) displays also mainly perinuclear staining. In both cases nuclei are spared. (*b*, phase contrast) Whole antiserum diluted 1:500 was used. Bar, 50 μm.

detect a relatively high rate of survival of ciliary ganglion neurons, indicating a strong CNTF-like activity. The cDNA-derived amino acid sequence of CNTF does not provide evidence for an aminoterminal signal sequence and CNTF is not released from transfected cells (35, 57). Thus CNTF is not thought to be secreted from cells. Furthermore, cultured Schwann cells express relatively low levels of CNTF mRNA as compared to astrocytes (57). These apparent discrepancies can be reconciled by the fact that the CNTF protein is relatively stable (53) and that high amounts of CNTF-like neurotrophic activity have been detected in lysates of cultured Schwann cells. Leakage from a small number of cells which are either damaged or are undergoing transient membrane disturbances might be sufficient to explain the observed survival activity. However, it cannot be excluded that CNTF is released via an alternative mechanism. Schwann cell conditioned media appear to contain additional neurotrophic activity for ciliary neurons which cannot be blocked by the CNTF-neutralizing antiserum. The molecule responsible for this effect is unknown so far. NGF and BDNF which are synthesized by Schwann cells are not active on ciliary neurons.

The release of CNTF-like activity by cultured Schwann cells has not been discussed in a recent study (1), wherein a polyclonal antiserum against mouse NGF was shown to substantially reduce survival activity of Schwann cell–conditioned media measured on dissociated chick E10 DRG neurons. This reduction was interpreted as a cross-reactivity of the antiserum with BDNF-like molecules. Spinal ganglion neurons of this age are, however, responsive to CNTF (5) and the presence of CNTF-like activity in supernatants of Schwann cells cultured under different conditions has been demonstrated (45, 63, and the present study). Thus it is not clear whether particular culture conditions can account for

the lack of CNTF-like activity in this study or whether the NGF-antiserum used may have effects in addition to the ones discussed by the authors. The chick nodose ganglion assay used in the present study for the detection of BDNF is highly sensitive to CNTF. Application of recombinant CNTF supports the survival of at least 40% of the plated neurons (52). Treatment of Schwann cell conditioned media with neutralizing anti-CNTF antibodies again demonstrated the effect of CNTF-like molecules in this assay. For these reasons, we decided to routinely include anti-CNTF antibodies in all nodose bioassays at a concentration high enough to reliably block 1 ng/ml of recombinant rat CNTF. The data obtained in the nodose bioassay are consistent with the assumption that Schwann cells secrete nearly saturating amounts of BDNF-like activity. Recently, the effects of Schwann cell conditioned media on the survival of E10 DRG neurons and on neurite outgrowth from retinal explants has been described which are in agreement with the present results (1). Others have also described neurotrophic activities of Schwann cell–conditioned media which could not be neutralized by NGF antisera. However, again the assay systems used did not exclude interference by CNTF (3, 45, 49, 63, 70).

Localization of BDNF-like Immunoreactivity in Cultured Schwann Cells

Immunostaining was used to provide further evidence for the presence of BDNF in Schwann cells. The homogenous staining of Schwann cells in pure or mixed cultures suggests that at least in culture all Schwann cells contribute to BDNF production. There seems to be a second cell type present at low abundance which is capable of synthesizing or internalizing BDNF-immunoreactive material. Sciatic fibroblasts, however, do not show detectable immunostaining. This finding is in agreement with the amounts of BDNF mRNA found in cultured sciatic fibroblasts which are so low that they can easily be explained by the presence of contaminating Schwann cells. This is in contrast to Northern blotting results obtained by others in cultures of rat dermal fibroblasts (1). These discrepancies might be due either to differential expression of BDNF in various fibroblast subpopulations or to contaminating cell types.

It is interesting to note that we detected an exclusively cytoplasmic immunoreactivity in Schwann cells. This is in contrast to the observations made on BDNF-synthesizing neurons in the CNS (67). Although the significance and structural basis of a potential nuclear localization of BDNF are not known, this finding demonstrates that nuclear localization is not an inevitable consequence of BDNF synthesis.

We conclude that there is good evidence that Schwann cells in culture and in lesioned peripheral nerves produce and secrete two different structurally related neurotrophins, NGF and BDNF. Production of neurotrophins might be beneficial for regenerating neurons. Although both neurotrophins act on spinal ganglion neurons their synthesis is differentially regulated in the nerve sheath, suggesting that the requirements for neurotrophins change with ongoing regeneration. It has been suggested that reexpression of the low affinity NGF receptor (p75) on Schwann cells in degenerating axons plays a role in the accumulation and presentation of NGF to regenerating axons (58). However, both NGF and BDNF bind to p75 (50). Considering the much slower dissociation from p75 of BDNF as compared to NGF (50), and the high concentrations of BDNF produced after nerve lesion, it is more likely that BDNF rather than NGF is being presented to regrowing axons.

Our results may partially explain the beneficial effects of peripheral nerve or Schwann cell grafts in CNS lesions. Such grafts allow partial regeneration and survival of retinal ganglion cells after optic nerve transsection (39, 64), regrowth of cholinergic fibers after fimbria-fornix lesions (19, 32, 66) and seem to support mesencephalic grafts (12, 17, 62). This interpretation is in agreement with in vitro studies showing that BDNF supports embryonic (31) and adult retinal ganglion cells (59), embryonic cholinergic (2), and dopaminergic neurons (30). Hence the success of these grafts might be explained by the combination of a permissive substrate (51) with an appropriate neurotrophic factor(s).

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