Synthesis and Localization of Ciliary Neurotrophic Factor in the Sciatic Nerve of the Adult Rat after Lesion and during Regeneration

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Abstract. Ciliary neurotrophic factor (CNTF) is expressed in high quantities in Schwann cells of peripheral nerves during postnatal development of the rat. The absence of a hydrophobic leader sequence and the immunohistochemical localization of CNTF within the cytoplasm of these cells indicate that the factor might not be available to responsive neurons under physiological conditions. However, CNTF supports the survival of a variety of embryonic neurons, including spinal motoneurons in culture. Moreover we have recently demonstrated that the exogenous application of CNTF protein to the lesioned facial nerve of the newborn rat rescued these motoneurons from cell death. These results indicate that CNTF might indeed play a major role in assisting the survival of lesioned neurons in the adult peripheral nervous system. Here we demonstrate that the CNTF mRNA and protein levels and the manner in which they are regulated are compatible with such a function in lesioned peripheral neurons. In particular, immunohistochemical analysis showed significant quantities of CNTF at extracellular sites after sciatic nerve lesion. Western blots and determination of CNTF biological activity of the same nerve segments indicate that extracellular CNTF seems to be biologically active. After nerve lesion CNTF mRNA levels were reduced to <5% in distal regions of the sciatic nerve whereas CNTF bioactivity decreased to only one third of the original before-lesion levels. A gradual reincrease in Schwann cells occurred concomitant with regeneration.

CILIARY neurotrophic factor (CNTF)[1] was originally characterized as a neurotrophic factor for cholinergic ciliary neurons, present in high quantities in putative target tissues, such as the embryonic chick eye (Helfand et al., 1976; Adler et al., 1979; Barbin et al., 1984). Subsequent studies have demonstrated that high levels of CNTF bioactivity are also present in the adult rat sciatic nerve (Williams et al., 1984; Manthorpe et al., 1986; Millaruelo et al., 1986). In addition to the effects of CNTF on ciliary neurons, it also affects the in vitro survival of a broad spectrum of neuronal cell types, such as neurons of sympathetic, dorsal root, trigeminal and nodose ganglia (Barbin et al., 1984; Eckenstein et al., 1990; Sendtner et al., 1991), and spinal motoneurons (Arakawa et al., 1990).

The final purification of CNTF protein from peripheral nerves of rabbit and rat and the cloning of the CNTF cDNA (Lin et al., 1989; Stöckli et al., 1989) suggested that the physiological function of this factor is most probably distinctly different from that of the neurotrophic factors of the nerve growth factor (NGF)-gene family (see Barde, 1990; Bothwell, 1991; Thoenen, 1991). In adult rats high levels of CNTF mRNA are found only in peripheral nerves and not in target tissues such as skeletal muscle or skin (Stöckli et al., 1989). Using specific anti-CNTF peptide anti-sera and CNTF mAbs, CNTF immunoreactivity within the adult rat sciatic nerve and iris is confined exclusively to Schwann cells (Stöckli et al., 1991; Sendtner et al., 1991; Rende et al., 1992). In the central nervous system a subpopulation of astrocytes seems to be the source of CNTF protein as shown immunohistochemically (Stöckli et al., 1991).

Although a variety of actions of CNTF have been characterized in vitro, very little is known about the physiological function of this protein in vivo. In newborn rats, where CNTF expression is still below the detection limit in peripheral nerves (Stöckli et al., 1989), most motoneurons undergo cell death after lesion within a short time period. We have demonstrated that exogenous application of CNTF can rescue lesioned motoneurons in neonatal rats with high efficacy (Sendtner et al., 1990). This observation suggests that CNTF might act as a lesion factor under pathophysiological conditions in the adult peripheral nervous system. However, it was not yet clear whether endogenous CNTF protein serves such a function after peripheral nerve lesion. Therefore, we have investigated the levels of CNTF mRNA, CNTF protein, and the location of CNTF immunoreactivity in different regions of the adult rat sciatic nerve after lesion and during regeneration. In the distal part of the lesioned

1. *Abbreviations used in this paper*: CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; TU, trophic unit.

sciatic nerve, CNTF mRNA is downregulated in Schwann cells after loss of axonal contact and re-expressed by the same cell type with ensuing regeneration. In contrast to the sharp decrease in CNTF mRNA to <5%, significant levels (~30% of control levels) of CNTF immunoreactivity and bioactivity are still detectable one week after lesion in these regions. At the lesion site, where the regeneration is initiated, significant levels of CNTF immunoreactivity are found in the extracellular space. These observations suggest that endogenous CNTF might indeed play a functional role after lesion of peripheral nerves.

Materials and Methods

Preparation of Sciatic Nerves

Sciatic nerve lesions in adult Wistar rats (~200 g body weight) of both sexes were performed as described by Heumann et al. (1987*a*). Each experiment was performed at least twice. Seven animals were used for each time point (tissues from four animals were pooled for RNA preparation and protein extracts were prepared from three animals). Additional rats were used for immunohistochemistry. Briefly, after anesthetizing rats with diethyl ether, the right sciatic nerve was cut or crushed at the level of the upper thigh. After transection both stumps were deflected to minimize regrowth of fibers. Crush lesions were carried out with forceps cooled in liquid nitrogen and the crush site was marked by a thread. After different time intervals the animals were killed by decapitation and segments were prepared from the lesioned and contralateral unlesioned nerve (see Figs. 1 *A* and 4 *A*). The segments were immediately frozen in liquid nitrogen and stored at −70°C before preparation of RNA or protein. For immunohistochemistry the rats were deeply anesthetized with ether and perfused transcardially with 4% formaldehyde in phosphate buffer as described previously (Stöckli et al., 1991). Similar segments were dissected from cut or crushed sciatic nerve as indicated.

Determination of CNTF mRNA Levels in Sciatic Nerve Segments

Total RNA from different regions of the lesioned and contralateral control sciatic nerves were isolated and processed for Northern blot according to Chomczynski and Sacchi (1987) as previously described (Stöckli et al., 1991): 26 picograms of a shortened synthetic CNTF-RNA standard covering the whole coding sequence of the CNTF mRNA (600 bp) were added to each tissue sample as a recovery standard before RNA extraction. After electrophoresis through a 1.4% agarose gel containing 2.0 M formaldehyde (Lehrach et al., 1977), RNA was vacuum blotted onto nylon membranes (Hybond-N, Amersham Corp., Arlington Heights, IL). CNTF standards (0.6 and 0.34 kb) were co-electrophoresed in separate lanes. A CNTF cRNA was prepared from a Bluescript SK+ vector containing the entire coding region of the cat CNTF cDNA (Stöckli et al., 1989). Hybridization conditions and autoradiography of the blots were performed as described previously by Stöckli et al. (1991). The determination of the absolute CNTF mRNA values was performed according to standard procedures (Heumann and Thoenen, 1986; Stöckli et al., 1991). Values of CNTF mRNA in Figs. 1 *B* and 4 *D* are expressed as a percentage of the values obtained from corresponding regions of the contralateral unlesioned nerve.

Western Blot Analysis of CNTF Protein

The preparation of nerve segments for bioassay and Western blot analysis was performed using published procedures (Stöckli et al., 1991). Briefly, the nerve segments were homogenized in a hypotonic phosphate buffer (5 mM, pH 7.0) containing 30 mM NaCl using a glass–glass homogenizer. After centrifugation for 30 min at 100,000 *g* in an ultracentrifuge (model TL-100; Beckman Instruments Inc., Palo Alto, CA) the clear supernatants were removed and the protein content determined by Coomassie-blue protein assay according to Bradford (1976) (BioRad Inc., Munich, Germany). 50 μg of protein were run per lane in 15% polyacrylamide gels under reducing conditions. Molecular mass markers (Lysozyme, 14.3 kD; Trypsinogen, 24 kD; and Ovalbumin 45 kD; 10 μg each) (Sigma Chemical Co., Deisenhofen, Germany) and 2 ng of recombinant rat CNTF (Masiakowski et al., 1991) were co-electrophoresed in separate lanes. After blotting to nitrocellulose membranes (Schleicher and Schüll Inc., Dassel, Germany) for 75 min at 150 mA according to Kyhse-Andersen (1984), using a semi-dry blotting apparatus (Fröbel Labortechnik, Lindau, Germany), the blots were blocked with TBS containing 5% horse serum and incubated with the monoclonal anti-CNTF antibody 4–68 (Stöckli et al., 1991) Hybridoma supernatant diluted 1:1 with TBS/5% horse serum overnight. The blots were then washed three times, blocked, and incubated with the second antibody (affinity-purified goat anti-mouse IgG [H+L] horseradish peroxidase conjugate) (BioRad Inc.) diluted 1:1,000 in TBS/5% horse serum. After three wash cycles with TBS the CNTF bands were visualized with chloronaphthol.

Bioassay for Neuronal Survival Activity

Ciliary neurons from 8-d-old chick embryos were prepared and cultured as described previously (Hughes et al., 1988). Briefly, after dissection and trypsinization of the ganglia, the cell suspension was preplated for enrichment of neuronal cells. The neurons thus obtained were cultured at a density of ~1,000 cells/well in polyornithine/laminin-coated 24-multi-well dishes (Costar Corp., Hialeah, FL) in F14 medium supplemented with 10% horse serum. Extracts from sciatic nerve segments were added at five different concentrations (10, 50, 150, 500, and 2,000 ng). The protein concentration (ng/ml) that supported half-maximal survival of the cultured neurons was defined as one trophic unit (TU).

Immunofluorescence

After dissection the lesioned sciatic nerves were postfixed for 2 h with 4% formaldehyde in phosphate buffer and dehydrated overnight with 30% sucrose. Frozen sections (7 μm) were prepared and dried on glass slides previously coated with gelatine, rehydrated overnight with 0.1 M Tris/PO4, pH 7.0, containing 0.1% gelatine and 0.2% Triton X-100 and incubated with the same CNTF mAb (4–68) as used for Western blot analysis (Hybridoma supernatant diluted 1:1 in the same buffer as described before) for 3 h. For double staining with a mouse anti-Neurofilament (200 kD) mAb (1:100, Boehringer Mannheim, Germany), a rabbit antiserum against recombinant rat CNTF was used at a 1:500 dilution. The sections were washed three times, incubated with second antibodies (affinity-purified biotinylated sheep anti-mouse Ig; Amersham Braunschweig, Germany) (in the case of the double staining, affinity purified anti-mouse Ig-Fluorescein, $F(ab)_2$, and an affinity-purified biotinylated sheep anti-rabbit $F(ab)_2$ were used at 1:100 dilutions [Boehringer Mannheim GmbH, Mannheim, Germany]) for 2 h, washed again, incubated with Texas red coupled to Streptavidin (Amersham Braunschweig; 1:100) and washed again three times. The tissue section was then incubated with TBS:Glycerin (1:1) and covered with glass cover slips before visualization under a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Controls were performed by adding excess recombinant CNTF (500 μg/ml) to the incubation step with the CNTF mAb, and 100 μg/ml in the case of the rabbit anti-CNTF antiserum (see Fig. 3, *h* and *i*). In these sections, the specific CNTF immunofluorescence could be completely abolished as shown previously for the 4–68 monoclonal anti-CNTF antibody (Stöckli et al., 1991).

Results

Effect of Transection on the CNTF mRNA and Protein Levels in Proximal and Distal Segments of the Sciatic Nerve

To investigate how CNTF expression is regulated in Schwann cells after loss of axonal contact, we cut the sciatic nerve of adult rats in the region of the upper thigh and inhibited regeneration by deflection of the proximal and distal stumps. 1 d after transection only a slight decrease in CNTF mRNA was observed in regions close to the site of lesion (Fig. 1 *B*). However, during the following 3 d CNTF mRNA levels were drastically reduced to <10% as compared with the unlesioned contralateral sciatic nerve. 1 wk after lesion the levels of CNTF mRNA fell to <5% compared with controls within distal parts of the lesioned nerve. A significant reduction of

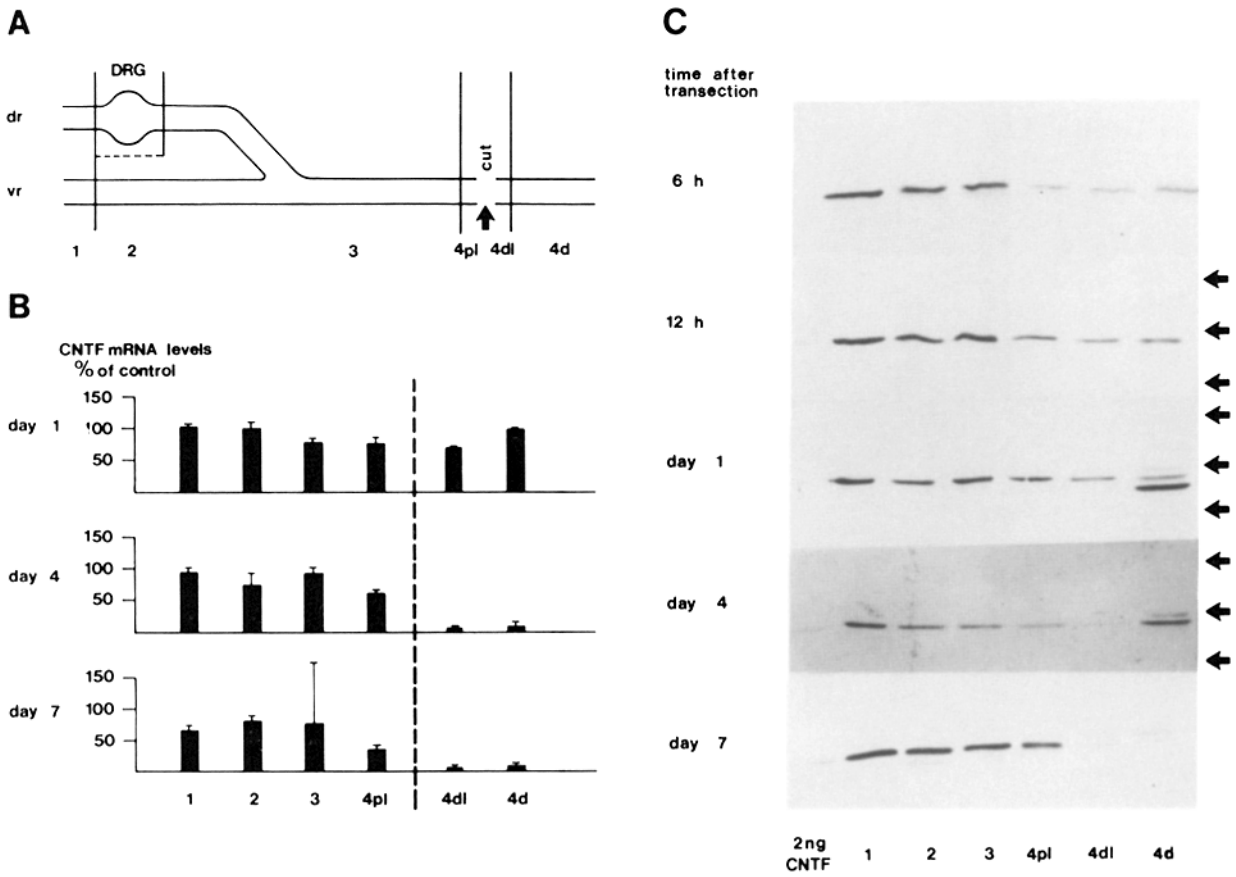

Figure 1. Levels of CNTF mRNA and immunoreactivity in various regions of transected sciatic nerve. (*A*) Schematic drawing of the regions analyzed. *dr*, dorsal root; *vr*, ventral root; *DRG*, dorsal root ganglion; *1*, cauda equina; *2*, DRG (L5); *3*, proximal sciatic nerve; *4pl*, a short 2-mm segment proximal to the lesion site; *4dl*, a 2-mm segment distal to the lesion site; and *4d*, distal sciatic nerve. (*B*) levels of CNTF mRNA in the nerve segments indicated in *A* at 1, 4, and 7 d after transection. Values represent average ±SEM of three independent determinations and are given as a percentage of the same segments of the contralateral unlesioned sciatic nerve. (*C*) Western blot analysis of CNTF protein in nerve segments at 6 and 12 h and 1, 4, and 7 d after nerve transection. 50 μg of soluble protein extract from the segments shown in *A* were loaded per one lane, 2 ng of recombinant rat CNTF was co-electrophoresed in a separate lane of each 15% polyacrylamide gel. CNTF immunoreactivity was detected by the mAb 4–68. Arrows shown together with the blots at 12 h and 1 and 4 d indicate the position of molecular mass markers coelectrophoresed in separate lanes and stained by amido black. Ovalbumin, 45 kD; trypsinogen, 24 kD; and lysozyme, 14.3 kD.

CNTF mRNA was also detectable in a short segment proximal to the lesion site (Fig. 1 *B*).

Western blots performed in parallel (Fig. 1 *C*) did not reveal marked quantitative changes in CNTF protein until 24 h after lesion. Distal regions of the sciatic nerve contained lower levels of CNTF immunoreactivity in accordance with earlier reports describing a twofold lower level of ciliary survival activity in distal regions as compared with proximal ones (Williams et al., 1984), most probably due to the fact that the cauda equina and the retroperitoneal nerves contain much less connective tissue as the peripheral parts of the nerves. However, qualitative changes in the Western blots became apparent in the distal nerve stump as early as 24 h after lesion: a major band with reduced molecular mass of ~20 kD was detectable in region 4d, in addition to other immunoreactive bands with lower molecular masses. Furthermore, a band of higher molecular mass (24 kD) became apparent. The intensity of the 24-kD immunoreactive band increased up to postlesion day 4, and was still detectable at significant intensity in both distal regions studied 1 wk after lesion. At 7 d after lesion, low, but nonetheless significant levels of CNTF immunoreactivity (at least 2 ng CNTF/50 μg of total extractable protein) were detectable within the distal part of the cut sciatic nerve.

The time course of changes in CNTF-immunoreactivity in Western blots of regions distal to the sciatic nerve cut site was paralleled by corresponding changes in ciliary neuronal survival activity (Fig. 2). During the first week after lesion there was a decrease in ciliary neuronal survival activity in both distal segments to ~30% of control levels. At day 7 ~2,000–3,000 TU/mg extractable protein were detectable in region 4d compared with 10,000 TU in the same region (region 4) of the unlesioned sciatic nerve.

To identify the location of the residual CNTF protein within the nerve, we performed immunohistochemical studies in various regions of the sciatic nerve 6 d after transection using either the same monoclonal CNTF antibody (4–68) as used for Western blots (Fig. 3, *b* and *d*) or a rabbit anti-CNTF antiserum (Fig. 3 *g*). In a region between 10 and 20 mm proximal to the lesion site (within region 3) most of the Schwann cells remained in contact with axons (Fig. 3 *a*). In these cells CNTF immunoreactivity was detectable exclu-

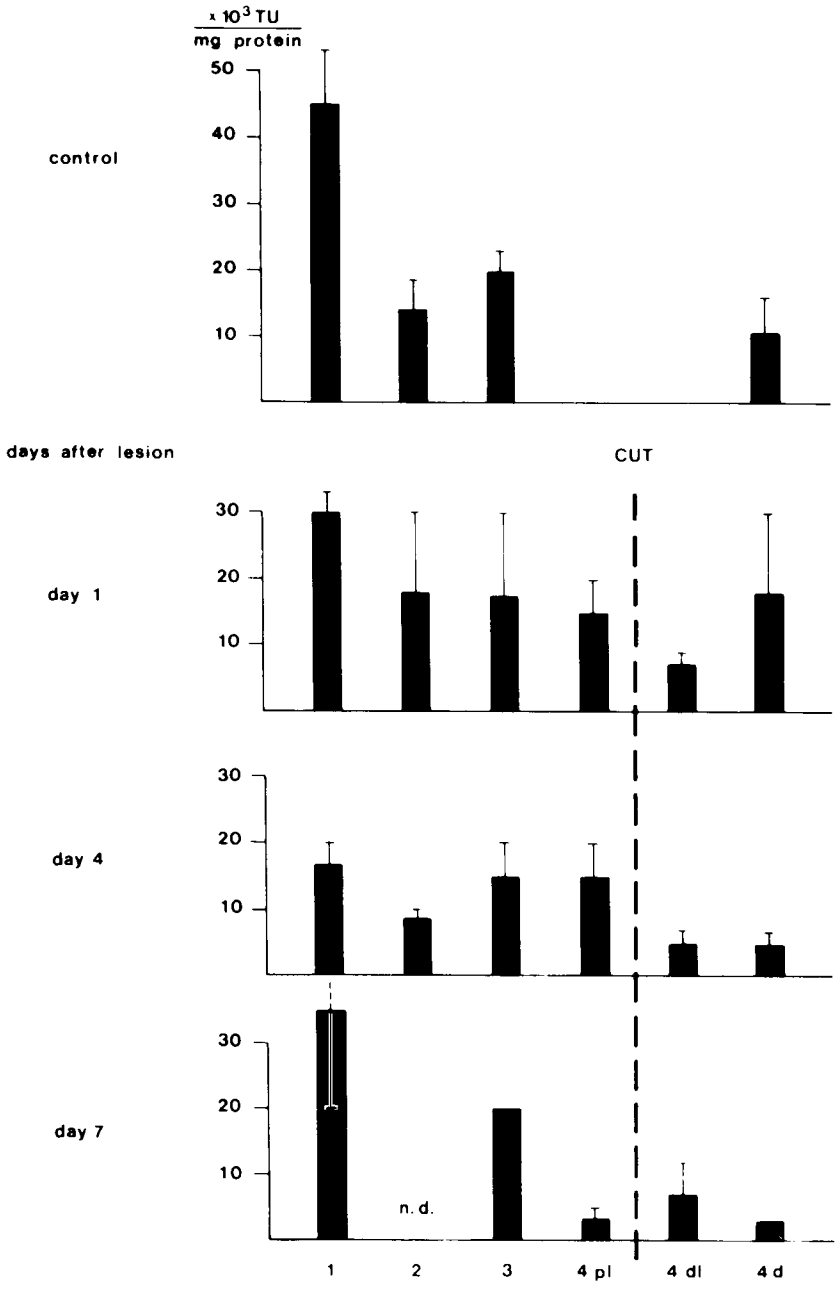

Figure 2. CNTF-bioactivity in segments of the unlesioned and transected sciatic nerve. Protein extracts from the same segments as shown in Fig. 1 were prepared as described in Materials and Methods and tested for their ability to support the survival of cultured ciliary neurons from 8-d-old chick embryos. Values are given in terms of trophic units as described in Materials and Methods and show the mean ± SD of two independent determinations.

sively in the cytoplasm (Fig. 3 *b*) similar to the situation of the unlesioned sciatic nerve (Stöckli et al., 1991). In contrast, within the proximal stump (region 4pl) most of the myelin sheaths were apparently degraded (Fig. 3 *c*). Only the few remaining myelinated Schwann cells (Fig. 3 *d*, *small arrow*) showed cytoplasmic CNTF immunoreactivity. In this region most of the CNTF immunoreactivity seemed to be associated with breakdown-products of myelin (Fig. 3, *c* and *d*, *bold arrows*). Within the distal stump (region 4d, Fig. 3, *e–i*) CNTF immunoreactivity also appeared at extracellular sites. Nearly all of the Schwann cells within this region lost their myelin sheaths (Fig. 3, *e* and *h*). Costaining with an anti-neurofilament (200 kD) mAb revealed axonal remnants still detectable 6 d after lesion (Fig. 3 *f*, *arrows*). CNTF immunoreactivity was detectable at the same location (Fig. 3 *g*, *arrows*) as these axonal fragments, indicating that it represents CNTF which was released from Schwann cells during the process of demyelination. Structures such as endoneurium or perineurium are unstained. Coincubation of a parallel section with excess CNTF (Fig. 3, *h* and *i*) during the incubation with the CNTF antiserum resulted in complete abolishment of the CNTF staining, demonstrating the specificity of the staining.

Levels of CNTF Expression during Regeneration of Lesioned Axons in the Rat Sciatic Nerve after Crush Lesion

The reduction in CNTF expression in the distal portion of the sciatic nerve after lesion raises the questions whether, when, and to what extent CNTF is re-expressed in Schwann cells during axonal regeneration. To address these questions, we performed crush lesions of the sciatic nerve and determined the levels of CNTF-mRNA and protein during the regeneration process.

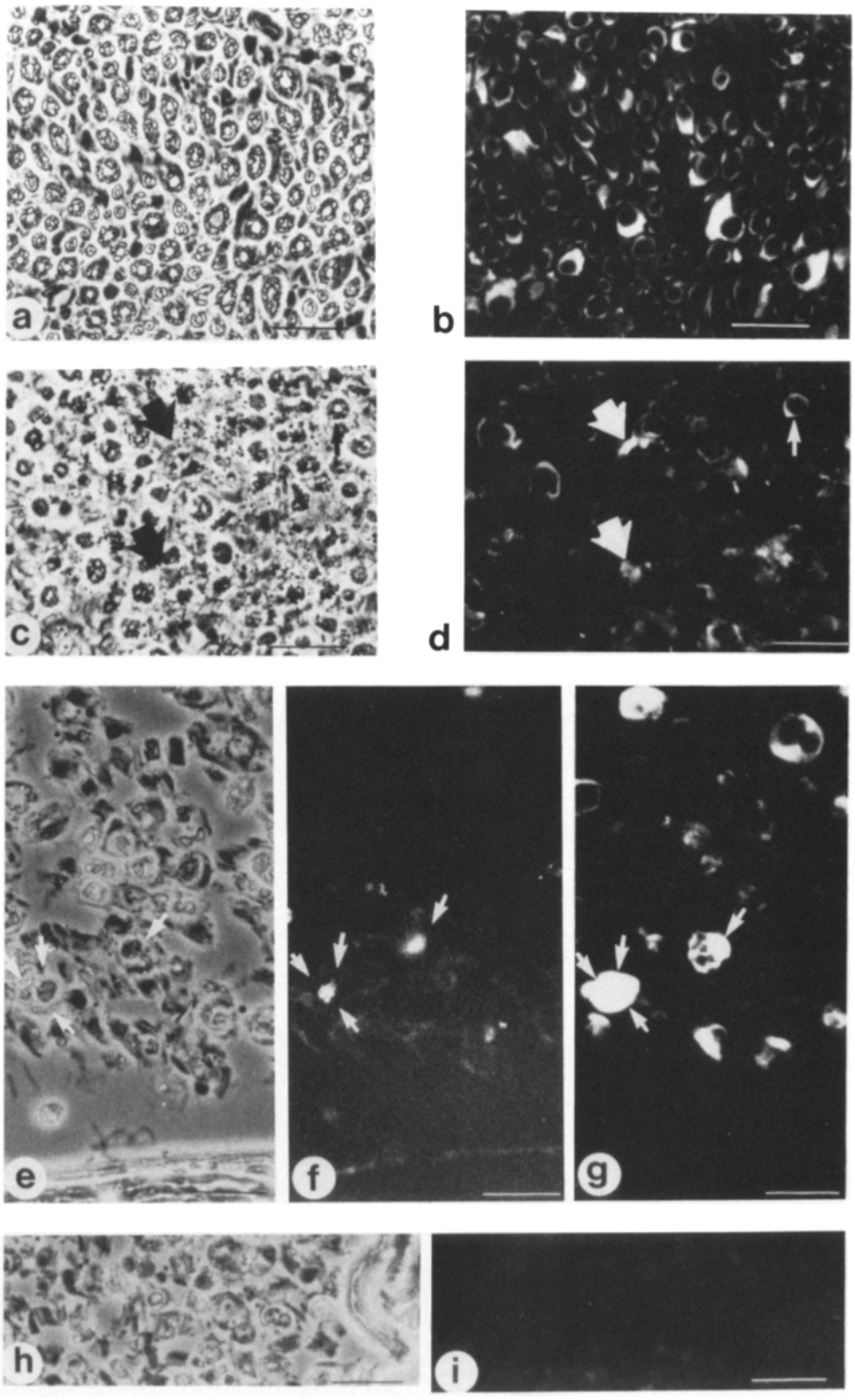

Figure 3. Immunolocalization of CNTF in sections of the sciatic nerve at 6 d after transection. *a*, *c*, *e*, and *h* show phase contrast pictures of (*a*) proximal segment (>20 mm proximal to the lesion site) (*c*) proximal stump; and (*e* and *h*) distal nerve segment (region 4d). *b*, *d*, and *g* show the corresponding CNTF immunoreactivity. (*h* and *i*) Parallel section of *e–g*, where 100 μg/ml CNTF were coincubated with the CNTF antiserum. Large arrows in *c* and *d* indicate the position of CNTF immunoreactive material at extracellular locations; the small arrows in *c* and *d* show CNTF immunoreactivity within the cytoplasm of a remaining Schwann cell; (*f*) Neurofilament staining of axonal remnants, and corresponding doublestaining against CNTF (*g*) reveals CNTF immunoreactivity within extracellular space (*arrows* in *e*, *f*, and *g*). Bar, 25 μm.

During the first 4 d after crush lesion similar results were obtained to those after transection of the sciatic nerve. 24 h after lesion a lower molecular mass band similar to that detected after transection became apparent in Western blots of extracts from a region distal to the crush site (Fig. 4 *B*) and an additional 24-kD CNTF-immunoreactive band was detectable at postlesion day 4 in both distal regions investigated (Fig. 4 *A*, region *4l* and *4d*).

The levels of CNTF-immunoreactivity (Fig. 4 *B*), bioactivity (Fig. 4 *C*), and mRNA (Fig. 4 *D*) dropped in a manner

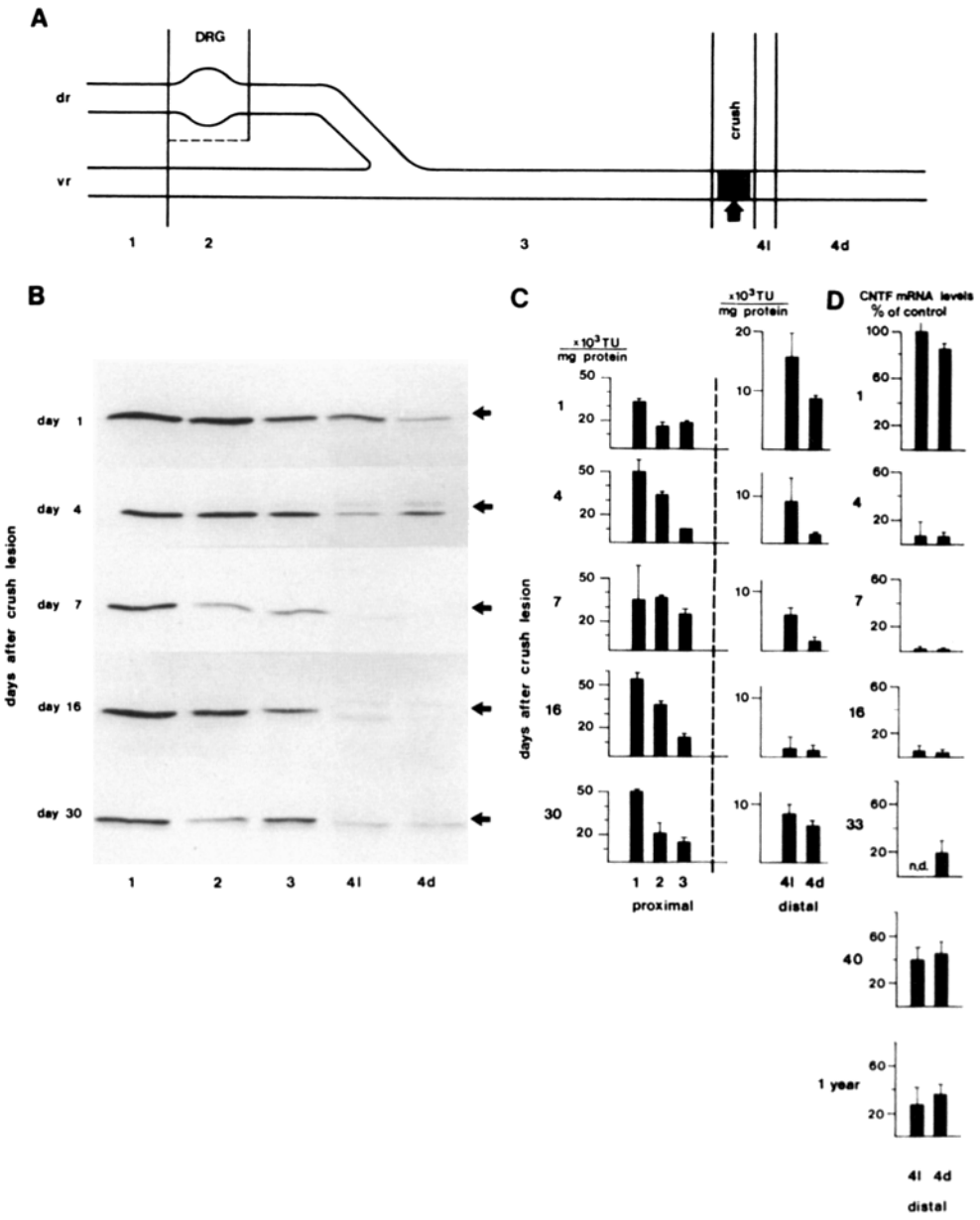

Figure 4. Levels of CNTF immunoreactivity, bioactivity, and mRNA after crush lesion of the adult rat sciatic nerve. (*A*) Schematic drawing of the sciatic nerve segments analyzed after crush lesion. *dr*, dorsal root; *vr*, ventral root; *DRG*, dorsal root ganglion; *1*, cauda equina; *2*, dorsal root ganglion (L5); *3*, proximal sciatic nerve; *4l*, 2-mm nerve segment distal to the crush site; and *4d*, distal sciatic nerve. (*B*) Western blot analysis of CNTF protein at 1, 4, 7, 16, and 30 d after nerve crush in the segments shown in *A*: 50 μg of soluble protein extract were loaded per lane. The CNTF immunoreactive bands were detected by the anti-CNTF mAb 4–68. Arrows indicate the position of the molecular mass marker trypsinogen (24 kD). (*C*) Ciliary neuronal survival activity in extracts from the same segments at 1, 4, 7, 16, and 30 d after nerve crush. Values are given as TU/mg protein extract and represent the mean ± SD of two independent experiments. A second scale is used for extracts from region 4l and 4d. (*D*) CNTF mRNA levels in distal segments of the crushed sciatic nerve at 1, 4, 7, 16, 33, and 40 d and 1 yr after lesion. Values represent the mean ± SEM of three independent determinations and are given as a percentage relative to similar regions of the contralateral unlesioned sciatic nerves.

similar to that seen after sciatic nerve transection. At 7 d after lesion reduced levels of CNTF immunoreactivity and bioactivity were detectable in regions distal to the crush site. Between 7 and 16 d after crush lesion the relative intensity of both the 22 kD and also the 24-kD CNTF-immunoreactive band increased in extracts of the segment 4l directly distal to the crush site in comparison with the more distal region 4d. (Fig. 4 *b*).

In Northern blots the levels of CNTF mRNA in sciatic nerve segments distally to the crush site dropped from slightly reduced levels at day 1 to levels of ~2% at day 7 in comparison with CNTF mRNA levels in the same segments of the unlesioned contralateral sciatic nerve (Fig. 4 *D*). At 16 d after crush in both distal regions a slight increase in CNTF mRNA levels was detectable. At 30 d after lesion a significant increase in CNTF-immunoreactivity and bioactivity was observed in both distal regions reaching levels between 30 and 50% of those in corresponding regions of unlesioned sciatic nerves. Similarly, from 16 to 33 d after crush CNTF mRNA levels in region 4d increased from 2.4 to 20% as compared with control levels, and up to day 40 these levels further increased to ~40%. However, no further increase was detectable after that time. The levels of CNTF mRNA in animals 1 yr after crush lesion of the sciatic nerve were not higher than those observed at 40 d after lesion.

CNTF-Immunoreactivity in Sections of Crushed Sciatic Nerve during Regeneration

To identify the individual cells which synthesize CNTF during regeneration we performed immunohistochemical studies at the crush site 6 and 10 d after lesion. At these time points many fibers had already regrown into the crush site and some fibers could already be detected morphologically (Figs. 5, *a–e*) or by neurofilament staining (data not shown) up to a few millimeters distal to the lesion site. Most of the

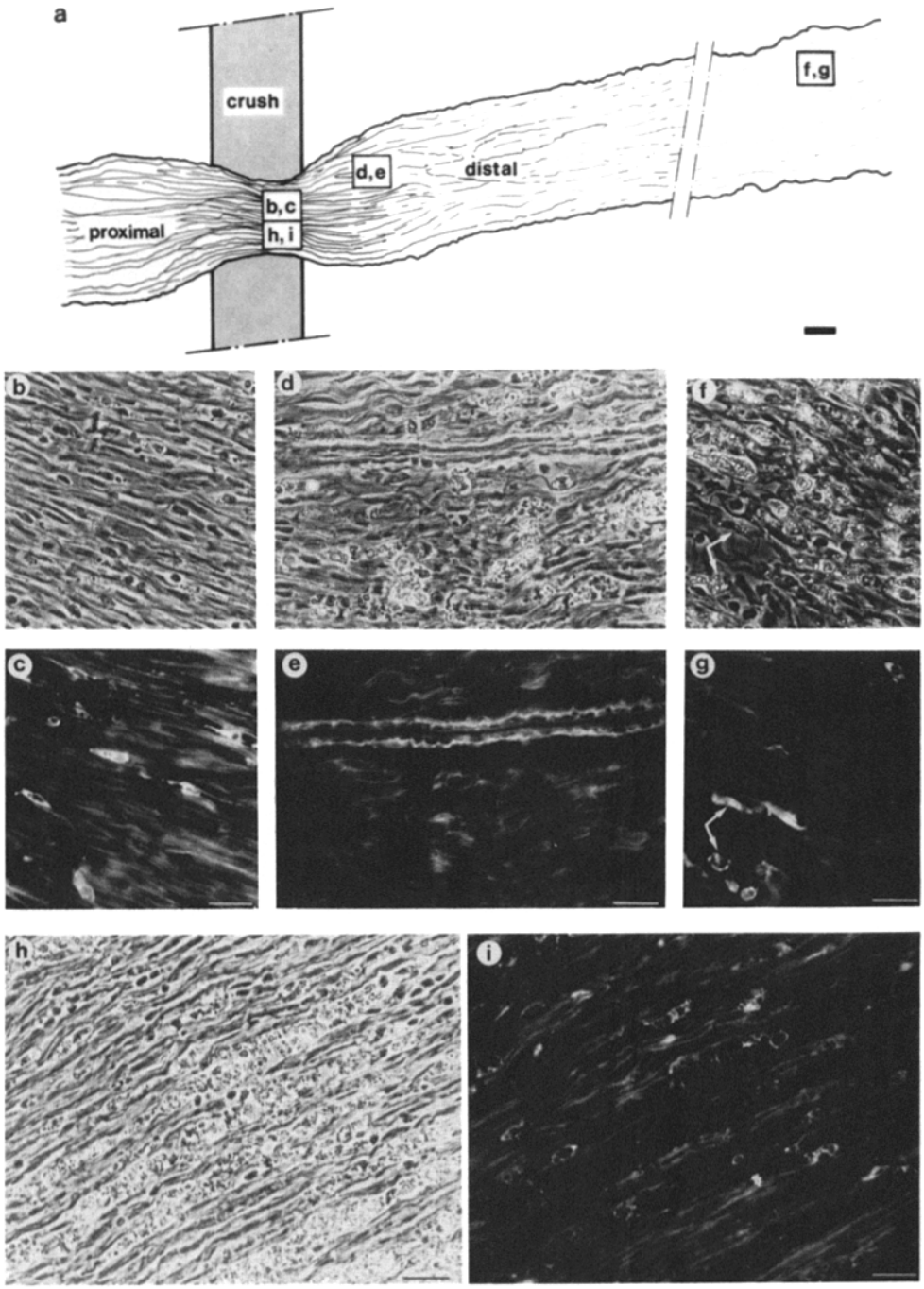

Figure 5. Immunolocalization of CNTF in the sciatic nerve 6 d (*b–g*) and 10 d (*h* and *i*) after crush lesion. (*a*) Schematic drawing of the crushed sciatic nerve indicating the positions where Fig. 5, *b–i* have been taken. (*b, d, f,* and *h*) High magnification phase-contrast fields, and (*c, e, g,* and *i*) corresponding CNTF-immunofluorescence. (*b* and *c*) Region from the same section shown in *a* within the distal part of the crush site. (*d* and *e*) Region from the same section as shown in *a* ~500 μm distal to the narrowest position of the crush site. (*f* and *g*) Region ~1.5 cm distal to the crush site. Arrows indicate CNTF-immunoreactivity associated with extracellular structures. In addition several cells within the same region, probably Schwann cells, show CNTF-immunoreactivity within their cytoplasm. (*h* and *i*) Comparable region to that shown in *b* and *c* 10 d after crush. Bars: (*a*) 200 μm; (*b–i*) 25 μm.

Schwann cells of this region had lost their myelin sheaths and their nuclei appeared to be round rather than showing the typical elongated features of myelinating Schwann cells (Fig. 5 *b*). Some of these Schwann cells were strongly stained for CNTF (Fig. 5, *b* and *c*), others were negative. 10 d after lesion, when Schwann cell proliferation in the same area has taken place, more cells with a round nucleus could be detected (Fig. 5, *h* and *i*). However, not all of these cells were CNTF positive and it was not clear whether all of the CNTF positive cells were in contact with regrowing nerve fibers. However, at a few locations within this nerve segment cells were detectable which apparently had been contacted again by regrowing axons and which were stained with high intensity against CNTF (Fig. 5, *d* and *e*). In a region further distal from the crush site where regeneration most probably had not yet occurred (Fig. 5, *f* and *g*) CNTF staining was detectable at extracellular sites, similar to the situation after transection. In addition, a few cells with intense CNTF immunoreactivity were present in the same region.

Discussion

The cloning of the CNTF cDNA (Stöckli et al., 1989; Lin et al., 1989; Masiakowski et al., 1991; Lam et al., 1991; Negro et al., 1991) and the determination of its regional and developmental expression (Stöckli et al., 1991) revealed that

CNTF does not fulfill the requirements of a target-derived neurotrophic factor. In particular, in the adult rat CNTF expression is not detectable in target tissues of responsive neurons, such as skeletal muscle and skin (Stöckli et al., 1989). The highest levels of CNTF mRNA are found in peripheral nerves (Stöckli et al., 1989) and immunohistochemically the CNTF protein is detectable in Schwann cells, both by CNTF peptide antisera and mAbs (Stöckli et al., 1991; Sendtner et al., 1991).

The finding that CNTF can rescue newborn rat facial motoneurons after axotomy with high efficiency (Sendtner et al., 1990) indicates that CNTF might be a lesion factor expressed by fully differentiated Schwann cells in the peripheral nervous system. After cut lesion of the adult rat sciatic nerve, precluding regeneration, CNTF mRNA levels decrease in the distal part of the sciatic nerve between 1 and 7 d after lesion. This time course coincides with that of axonal degeneration in the distal part of the lesioned sciatic nerve in adult rodents (Brown et al., 1991), indicating that Schwann cells stop synthesizing CNTF after loss of axonal contact. However, in contrast to the results for CNTF mRNA, CNTF immunoreactivity and biological activity are still detectable in significant quantities for at least 7 d after lesion within the distal parts of the lesioned sciatic nerve. In terms of ciliary neuronal survival activity, more than 1,000 TU are still detectable per one milligram of protein extract in distal nerve segments after lesion (Fig. 2). A strong immunoreactive band became apparent in Western blots of region 4d at 4 d after cut (Fig. 1) or crush lesion (Fig. 4). At 7 d after lesion, 50 μg of protein extract from the same region still showed a CNTF-immunoreactive band, which corresponds in its intensity to at least 2 ng of recombinant CNTF (Fig. 1). This would correspond to 40 ng CNTF/mg of soluble protein extracted from this part of the lesioned nerve. Although these levels are about three to four times lower than those of the unlesioned sciatic nerve (Fig. 2) they might nonetheless be of essential functional importance given the high potency of CNTF. CNTF-immunoreactivity in uninjured peripheral nerves is detectable only within the cytoplasm of intact Schwann cells (Stöckli et al., 1991; Sendtner et al., 1991; Rende et al., 1992). This finding suggests that in the intact nerve CNTF is either not available to neurons or is present at extracellular sites in only very low quantities, which cannot be detected by immunohistochemical procedures. Interestingly, in the lesioned sciatic nerve a significant proportion of the CNTF-immunoreactivity is detectable in the extracellular space and not in the cytoplasm of intact Schwann cells. The CNTF protein detectable at these sites seems to be available in biologically active form for regenerating neurons which grow into these distal parts of the lesioned nerve.

It has to be taken into consideration that other factors, such as acidic and basic FGF (aFGF and bFGF), might account (at least partially) for the ciliary survival activity in protein extracts of the lesioned sciatic nerve. Both factors have been described as being able to increase the survival of ciliary neurons in culture (Unsicker et al., 1987; Watters and Hendry, 1987; Eckenstein et al., 1990). Of these two factors only aFGF seems to be present in relatively high quantities in the adult rat sciatic nerve (Eckenstein et al., 1991) and it has been shown (Elde et al., 1991) that aFGF is located on the cytoplasmic side of axonal membranes. However, within 3 d after lesion the extractable biological activity attributed to FGF is reduced to <10% of control (Eckenstein et al., 1991). Significantly less than 100 mitotic units per milligram of extractable protein (1 mitotic unit corresponds to 100–200 picograms of aFGF or bFGF, the same concentrations which have been shown by Eckenstein et al. (1990) to support half-maximal survival of cultured ciliary neurons) have been reported to be preserved in the distal region of the lesioned sciatic nerve. If these data are compared with our results concerning the levels of CNTF mRNA and immunoreactivity in Western blots and in tissue sections, major differences between aFGF and CNTF are apparent: aFGF is found within the axons and CNTF within the cytoplasm of Schwann cells in the adult rat sciatic nerve. The levels of aFGF are reduced to <10% of control within 3 d after lesion, whereas CNTF bioactivity levels are distinctly higher (Fig. 2) under comparable conditions. Moreover, CNTF immunoreactivity is still detectable in substantial quantities 6 d after lesion within the extracellular space (Fig. 3) of the lesioned nerve. The detection of CNTF protein by Western blot (Fig. 1) and ciliary neuronal survival activity (Fig. 2) indicates that this immunostaining seems to reflect biologically active CNTF.

Western blot analysis of different segments of the sciatic nerve after cut or crush lesion shows that a significant post-translational modification of CNTF protein seems to occur after lesion. 24 h after lesion, a second CNTF-immunoreactive band with a molecular mass of ~24 kD becomes detectable in segments distal to the lesion site. This second immunoreactive band increases in relative intensity up to 7 d after lesion and then gradually decreases to barely detectable levels by day 30, when regenerating axons have reached this area. This 24-kD band might reflect posttranslationally modified CNTF (the molecular nature of this modification is not clear) which is released from Schwann cells during demyelination caused by axonal degeneration. This interpretation is supported by the finding that this second CNTF-immunoreactive band is also detectable in Western blots of sciatic nerve extracts of Wobbler mice (Kaupmann et al., 1991*b*). In this mouse mutant axonal degeneration of motoneurons and secondary demyelination of peripheral nerves results from a genetic defect (Duchen et al., 1968; Kaupmann et al., 1991*a*) and occurs without mechanical nerve lesion.

The changes in CNTF protein and mRNA levels after lesion of the rat sciatic nerve are distinctly different from those observed for NGF and its mRNA (Richardson and Ebendahl, 1982; Korsching and Thoenen, 1983; Heumann et al., 1987*a*). After sciatic nerve lesion (cut or crush) there is a rapidly occurring, but also long lasting, elevation of NGF protein. During the first few hours after lesion the increase in NGF protein results from the accumulation of retrogradely transported NGF (Korsching and Thoenen, 1983) which is then followed by enhanced local synthesis by non-neuronal cells of the lesioned nerve (Heumann et al., 1987*a*,*b*; Lindholm et al., 1987). In addition to the lesion-mediated increase in NGF in the sciatic nerve there is also an upregulation of the low affinity (P75) NGF receptor in Schwann cells in the region of the degenerating axons (Taniuchi et al., 1986; Heumann et al., 1987*b*; Raivich and Kreutzberg, 1987; Raivich et al., 1991). To some extent the upregulation of the P75 receptor is reciprocal to that of

CNTF. However, the regulation of the low affinity NGF receptor is more precisely regulated by axonal contact than is the case for CNTF. As soon as regenerating nerve fibers come into contact with Schwann cells the P75 receptors are immediately downregulated. In contrast, CNTF immunoreactivity appears not only in those Schwann cells which are in immediate contact with regenerating axons but also in a few Schwann cells in more distal regions which most probably have not yet been reached by regenerating nerve fibers (Fig. 5 *g*).

In summary, we have shown that significant quantities of CNTF protein are detectable at extracellular sites in distal parts of the lesioned sciatic nerve when CNTF mRNA levels are already very low. The CNTF protein found in these locations is biologically active. Thus, it seems to be available to regenerating axons and accordingly fulfills the requirements of a lesion factor. The high levels of CNTF expression in differentiated Schwann cells are only maintained as long as these cells are in contact with axons. However, axonal contact is apparently not the only mechanism which regulates CNTF expression in Schwann cells, as strongly labeled Schwann cells can also be detected in regions of the lesioned nerve which have not yet been reached by regenerating nerve fibers. The results of the present investigation are compatible with the assumption that CNTF acts as a lesion factor in the adult. However, it remains to be demonstrated as to whether CNTF is released under physiological conditions in small quantities by a not yet identified release mechanism and also plays a role in maintaining the normal function of responsive neurons, in particular, motoneurons. To answer this question the elimination of the CNTF gene by homologous recombination seems to be a promising approach to elucidate the possible physiological and the pathophysiological role played by CNTF in comparison with other neurotrophic molecules.

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